CORONAVIRUS DISEASE (COVID-19): PATHOPHYSIOLOGY, EPIDEMIOLOGY, CLINICAL MANAGEMENT AND PUBLIC HEALTH RESPONSE

EDITED BY: Zisis Kozlakidis, Denise L. Doolan, Shen-Ying Zhang, Yasuko Tsunetsugu Yokota, Tatsuo Shioda, Rukhsana Ahmed, Mohan Jyoti Dutta, Ata Murat Kaynar, Michael Kogut, Hannah Bradby, Slobodan Paessler, Alex Rodriguez-Palacios, Alexis M. Kalergis, Longxiang Su, Abdallah Samy and Zhongheng Zhang

PUBLISHED IN: Frontiers in Public Health, Frontiers in Immunology, Frontiers in Big Data, Frontiers in Artificial Intelligence, Frontiers in Cellular and Infection Microbiology, Frontiers in Physics, Frontiers in Neurology, Frontiers in Microbiology, Frontiers in Pediatrics, Frontiers in Medicine, Frontiers in Cell and Developmental Biology, Frontiers in Veterinary Science, Frontiers in Communication and Frontiers in Sociology

frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-83252-230-1
DOI 10.3389/978-2-83252-230-1

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: frontiersin.org/about/contact

CORONAVIRUS DISEASE (COVID-19): PATHOPHYSIOLOGY, EPIDEMIOLOGY, CLINICAL MANAGEMENT AND PUBLIC HEALTH RESPONSE

Citation: Kozlakidis, Z., Doolan, D. L., Zhang, S.-Y., Yokota, Y. T., Shioda, T., Ahmed, R., Dutta, M. J., Kaynar, A. M., Kogut, M., Bradby, H., Paessler, S., Rodriguez-Palacios, A., Kalergis, A. M., Su, L., Samy, A., Zhang, Z., eds. (2023). Coronavirus Disease (COVID-19): Pathophysiology, Epidemiology, Clinical Management and Public Health Response. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-83252-230-1

Table of Contents

13 ***COVID-19 Clinical Characteristics, and Sex-Specific Risk of Mortality: Systematic Review and Meta-Analysis***
Mohammad Javad Nasiri, Sara Haddadi, Azin Tahvildari, Yeganeh Farsi, Mahta Arbabi, Saba Hasanzadeh, Parnian Jamshidi, Mukunthan Murthi and Mehdi Mirsaeidi

23 ***Mechanisms Underlying Potential Therapeutic Approaches for COVID-19***
Abdelouaheb Benani and Sanae Ben Mkaddem

32 ***The Long Road Toward COVID-19 Herd Immunity: Vaccine Platform Technologies and Mass Immunization Strategies***
Lea Skak Filtenborg Frederiksen, Yibang Zhang, Camilla Foged and Aneesh Thakur

58 ***Coronavirus (SARS-CoV-2) and Mortality Rate in India: The Winning Edge***
Gyaneshwer Chaubey

63 ***Association Between ABO Blood Group System and COVID-19 Susceptibility in Wuhan***
Qian Fan, Wei Zhang, Bo Li, De-Jia Li, Jian Zhang and Fang Zhao

70 ***Emergency Response Measures for Anesthesia Nursing During the COVID-19 Pandemic: West China Hospital Experiences***
Ping Zheng, Ruihao Zhou, Lu Yin, Xiaorong Yin, Yongqiao Mao, Heng Wang, Ling Ye and Tao Zhu

78 ***Geographic and Genomic Distribution of SARS-CoV-2 Mutations***
Daniele Mercatelli and Federico M. Giorgi

91 ***Dissemination Strategies and Usage of Psychological Assistance Hotlines During the COVID-19 Outbreak in China***
Ruofan Ma, Rin Nguyen and Jonathan M. Oakman

104 ***Quinolines-Based SARS-CoV-2 3CLpro and RdRp Inhibitors and Spike-RBD-ACE2 Inhibitor for Drug-Repurposing Against COVID-19: An* in silico *Analysis***
Rajaiah Alexpandi, Joelma Freire De Mesquita, Shunmugiah Karutha Pandian and Arumugam Veera Ravi

119 ***Novel Criteria for When and How to Exit a COVID-19 Pandemic Lockdown***
Chenyu Li, Paola Romagnani and Hans-Joachim Anders

124 ***Assessment of Healthcare System Capabilities and Preparedness in Yemen to Confront the Novel Coronavirus 2019 (COVID-19) Outbreak: A Perspective of Healthcare Workers***
Mohammed Zawiah, Fahmi Y. Al-Ashwal, Ramzi Mukred Saeed, Mohammed Kubas, Sara Saeed, Amer Hayat Khan, Syed Azhar Syed Sulaiman and Rami Abduljabbar

132 ***Racial and Gender-Based Differences in COVID-19***
Jonathan Kopel, Abhilash Perisetti, Ali Roghani, Muhammad Aziz, Mahesh Gajendran and Hemant Goyal

140 ***Misconceptions on COVID-19 Risk Among Ugandan Men: Results From a Rapid Exploratory Survey, April 2020***
Keneth Iceland Kasozi, Ewan MacLeod, Fred Ssempijja, Michael W. Mahero, Kevin Matama, Grace Henry Musoke, Kevin Bardosh, Robinson Ssebuufu, Florence Wakoko-Studstil, Isaac Echoru, Emmanuel Tiyo Ayikobua, Regan Mujinya, Grace Nambuya, Hope Onohuean, Gerald Zirintunda, Justine Ekou and Susan Christina Welburn

150 ***Spironolactone: An Anti-androgenic and Anti-hypertensive Drug That May Provide Protection Against the Novel Coronavirus (SARS-CoV-2) Induced Acute Respiratory Distress Syndrome (ARDS) in COVID-19***
Flavio A. Cadegiani, Carlos G. Wambier and Andy Goren

155 ***Improving Non-specific Immunity to Coronavirus Disease (COVID-19) by the Novelty, Diversity, and Quantity of Antigen***
Patrice Boucher and Roger Boucher

158 ***Healthcare Transformation in the Post-Coronavirus Pandemic Era***
Abdul Rahman Jazieh and Zisis Kozlakidis

164 ***Contriving Multi-Epitope Subunit of Vaccine for COVID-19: Immunoinformatics Approaches***
Rong Dong, Zhugang Chu, Fuxun Yu and Yan Zha

182 ***Efficacy and Safety of Anti-malarial Drugs (Chloroquine and Hydroxy-Chloroquine) in Treatment of COVID-19 Infection: A Systematic Review and Meta-Analysis***
Rashmi Ranjan Das, Nishant Jaiswal, Nishanth Dev, Nikita Jaiswal, Sushree Samiksha Naik and Jhuma Sankar

198 ***Culture-Centered Processes of Community Organizing in COVID-19 Response: Notes From Kerala and Aotearoa New Zealand***
Mohan J. Dutta, Christine Elers and Pooja Jayan

213 ***COVID-19: A Multidisciplinary Review***
Nour Chams, Sana Chams, Reina Badran, Ali Shams, Abdallah Araji, Mohamad Raad, Sanjay Mukhopadhyay, Edana Stroberg, Eric J. Duval, Lisa M. Barton and Inaya Hajj Hussein

233 ***CoronaVR: A Computational Resource and Analysis of Epitopes and Therapeutics for Severe Acute Respiratory Syndrome Coronavirus-2***
Amit Kumar Gupta, Md. Shoaib Khan, Shubham Choudhury, Adhip Mukhopadhyay, Sakshi, Amber Rastogi, Anamika Thakur, Pallawi Kumari, Manmeet Kaur, Shalu, Chanchal Saini, Vandna Sapehia, Barkha, Pradeep Kumar Patel, Kailash T. Bhamare and Manoj Kumar

254 ***Treatment Options for COVID-19: A Review***
Mukarram Jamat Ali, Muhammad Hanif, Muhammad Adnan Haider, Muhammad Umer Ahmed, FNU Sundas, Arham Hirani, Izhan Ali Khan, Khurram Anis and Amin H. Karim

264 ***Addressing COVID-19 Communication and Management by a Systems Thinking Approach***
Francesco Gonella, Marco Casazza, Silvio Cristiano and Alessandra Romano

272 ***Quarantine Due to the COVID-19 Pandemic From the Perspective of Pediatric Patients With Type 1 Diabetes: A Web-Based Survey***
Stefano Passanisi, Maria Pecoraro, Francesco Pira, Angela Alibrandi, Vittoria Donia, Paola Lonia, Giovanni Battista Pajno, Giuseppina Salzano and Fortunato Lombardo

278 ***D-Dimer Concentrations and COVID-19 Severity: A Systematic Review and Meta-Analysis***

Panagiotis Paliogiannis, Arduino Aleksander Mangoni, Paola Dettori, Gheyath K. Nasrallah, Gianfranco Pintus and Angelo Zinellu

285 ***Ruxolitinib Rapidly Reduces Acute Respiratory Distress Syndrome in COVID-19 Disease. Analysis of Data Collection From RESPIRE Protocol***

Enrico Capochiani, Bruno Frediani, Giorgio Iervasi, Aldo Paolicchi, Spartaco Sani, Paolo Roncucci, Annarosa Cuccaro, Federico Franchi, Federico Simonetti, Davide Carrara, Ilaria Bertaggia, Daniela Nasso, Rossella Riccioni, Sabino Scolletta, Serafina Valente, Edoardo Conticini, Alessandro Gozzetti and Monica Bocchia

294 ***Saliva as a Candidate for COVID-19 Diagnostic Testing: A Meta-Analysis***

László Márk Czumbel, Szabolcs Kiss, Nelli Farkas, Iván Mandel, Anita Hegyi, Ákos Nagy, Zsolt Lohinai, Zsolt Szakács, Péter Hegyi, Martin C. Steward and Gábor Varga

304 ***Directly Acting Antivirals for COVID-19: Where Do We Stand?***

Siew L. Teoh, Yi H. Lim, Nai M. Lai and Shaun W. H. Lee

322 ***Quantitative Assessment of Parenchymal Involvement Using 3D Lung Model in Adolescent With Covid-19 Interstitial Pneumonia***

Luca Borro, Paolo Ciliberti, Teresa Pia Santangelo, Andrea Magistrelli, Andrea Campana, Francesca Calò Carducci, Marabotto Caterina, Paolo Tomà and Aurelio Secinaro

326 ***Child Healthcare and Immunizations in Sub-Saharan Africa During the COVID-19 Pandemic***

Danilo Buonsenso, Bianca Cinicola, Memenatu Ngaima Kallon and Francesco Iodice

330 ***Value of Viral Nucleic Acid in Sputum and Feces and Specific IgM/IgG in Serum for the Diagnosis of Coronavirus Disease 2019***

Yuwen He, Jiangyan Luo, Jie Yang, Jinlong Song, Li Wei and Weifeng Ma

336 ***Clinical Characteristics and Short-Term Outcomes of Severe Patients With COVID-19 in Wuhan, China***

Xiaobo Feng, Peiyun Li, Liang Ma, Hang Liang, Jie Lei, Wenqiang Li, Kun Wang, Yu Song, Shuai Li, Wei Yang and Cao Yang

348 ***Case Report: Benign Infantile Seizures Temporally Associated With COVID-19***

Marcos García-Howard, Mercedes Herranz-Aguirre, Laura Moreno-Galarraga, María Urretavizcaya-Martínez, Josune Alegría-Echauri, Nerea Gorría-Redondo, Laura Planas-Serra, Agatha Schlüter, Marta Gut, Aurora Pujol and Sergio Aguilera-Albesa

353 ***Convalescent Plasma: A Potential Life-Saving Therapy for Coronavirus Disease 2019 (COVID-19)***

Ahmed N. Alghamdi and Ahmed S. Abdel-Moneim

357 ***Overview of Immune Response During SARS-CoV-2 Infection: Lessons From the Past***

Vibhuti Kumar Shah, Priyanka Firmal, Aftab Alam, Dipyaman Ganguly and Samit Chattopadhyay

374 ***Potential Effect of COVID-19 on Maternal and Infant Outcome: Lesson From SARS***

Yun Wang, Yiliang Wang, Xiaoxue Han, Jiazhuo Ye and Ruiman Li

383 ***Plants Metabolites: Possibility of Natural Therapeutics Against the COVID-19 Pandemic***
Farhana Rumzum Bhuiyan, Sabbir Howlader, Topu Raihan and Mahmudul Hasan

409 ***COVID-19 Consumer Health Information Needs Improvement to Be Readable and Actionable by High-Risk Populations***
Alison Caballero, Katherine Leath and Jamie Watson

416 ***Remodeling of the Immune Response With Aging: Immunosenescence and Its Potential Impact on COVID-19 Immune Response***
Lucas Leite Cunha, Sandro Felix Perazzio, Jamil Azzi, Paolo Cravedi and Leonardo Vidal Riella

427 ***Early Epidemiological Features of COVID-19 in Nepal and Public Health Response***
Santosh Dhakal and Surendra Karki

435 ***Understanding the Pathophysiology of COVID-19: Could the Contact System Be the Key?***
Simone Meini, Andrea Zanichelli, Rodolfo Sbrojavacca, Federico Iuri, Anna Teresa Roberts, Chiara Suffritti and Carlo Tascini

444 ***Clinical Characteristics and Prognosis of 218 Patients With COVID-19: A Retrospective Study Based on Clinical Classification***
Xiquan Yan, Xiaotong Han, Danhong Peng, Yong Fan, Zhixiong Fang, Da Long, Yu Xie, Shuibo Zhu, Fang Chen, Wei Lin and Yimin Zhu

455 ***Pulmonary and Extra-Pulmonary Clinical Manifestations of COVID-19***
Kemmian D. Johnson, Christen Harris, John K. Cain, Cicily Hummer, Hemant Goyal and Abhilash Perisetti

467 ***Modeling the Onset of Symptoms of COVID-19***
Joseph R. Larsen, Margaret R. Martin, John D. Martin, Peter Kuhn and James B. Hicks

481 ***A Simple Bayesian Method for Evaluating Whether Data From Patients With Rheumatic Diseases Who Have Been Under Chronic Hydroxychloroquine Medication Since Before the COVID-19 Outbreak Can Speak to Hydroxychloroquine's Prophylactic Effect Against Infection With SARS-CoV-2***
Serban C. Musca

487 ***Antivirals Against Coronaviruses: Candidate Drugs for SARS-CoV-2 Treatment?***
Igor de Andrade Santos, Victória Riquena Grosche, Fernando Rodrigues Goulart Bergamini, Robinson Sabino-Silva and Ana Carolina Gomes Jardim

510 ***Vaccine Development Against COVID-19 Prior to Pandemic Outbreaks, Using* in vitro *Evolution and Reverse Genetics***
Hatem Zayed

513 ***Bibliometric Analysis on COVID-19: A Comparison of Research Between English and Chinese Studies***
Jingchun Fan, Ya Gao, Na Zhao, Runjing Dai, Hailiang Zhang, Xiaoyan Feng, Guoxiu Shi, Jinhui Tian, Che Chen, Brett D. Hambly and Shisan Bao

523 ***Performance of Two Risk-Stratification Models in Hospitalized Patients With Coronavirus Disease***
Rong Xu, Keke Hou, Kun Zhang, Huayan Xu, Na Zhang, Hang Fu, Linjun Xie, Ran Sun, Lingyi Wen, Hui Liu, Zhigang Yang, Ming Yang and Yingkun Guo

530 ***Themes and Evolution of Misinformation During the Early Phases of the COVID-19 Outbreak in China—An Application of the Crisis and Emergency Risk Communication Model***
Jiahui Lu

537 ***Viral Transmission and Clinical Features in Asymptomatic Carriers of SARS-CoV-2 in Wuhan, China***
Fen Tan, Kaige Wang, Jiasheng Liu, Dan Liu, Jianfei Luo and Rui Zhou

542 ***Analysis of the Virus Contamination and Disinfection Effect in Isolation Ward of Patients With COVID-19***
Shiyang Zhang, Chuanpeng Wang, Minqiang Lin, Qinsheng Deng, Yuzhen Ye, Zhiyong Li, Lixin Qiu and Zhanxiang Wang

547 ***COVID-19 Pandemic: Group Testing***
Ozkan Ufuk Nalbantoglu and Aycan Gundogdu

552 ***Artificial Intelligence for COVID-19 Drug Discovery and Vaccine Development***
Arash Keshavarzi Arshadi, Julia Webb, Milad Salem, Emmanuel Cruz, Stacie Calad-Thomson, Niloofar Ghadirian, Jennifer Collins, Elena Diez-Cecilia, Brendan Kelly, Hani Goodarzi and Jiann Shiun Yuan

565 ***The Strengths of Scanning Electron Microscopy in Deciphering SARS-CoV-2 Infectious Cycle***
Djamal Brahim Belhaouari, Anthony Fontanini, Jean-Pierre Baudoin, Gabriel Haddad, Marion Le Bideau, Jacques Yaacoub Bou Khalil, Didier Raoult and Bernard La Scola

576 ***Exploring the Demographics and Clinical Characteristics Related to the Expression of Angiotensin-Converting Enzyme 2, a Receptor of SARS-CoV-2***
Shengjie Li, Jianping Han, Aiping Zhang, Yi Han, Miaomiao Chen, Zhenzhen Liu, Mingxi Shao and Wenjun Cao

585 ***Single-Cell RNA-seq Identifies Cell Subsets in Human Placenta That Highly Expresses Factors Driving Pathogenesis of SARS-CoV-2***
Nancy Ashary, Anshul Bhide, Priyanka Chakraborty, Stacy Colaco, Anuradha Mishra, Karisma Chhabria, Mohit Kumar Jolly and Deepak Modi

601 ***The 2020 Pandemic: Current SARS-CoV-2 Vaccine Development***
Sana O. Alturki, Sawsan O. Alturki, Jennifer Connors, Gina Cusimano, Michele A. Kutzler, Abdullah M. Izmirly and Elias K. Haddad

614 ***SARS-CoV-2 Codon Usage Bias Downregulates Host Expressed Genes With Similar Codon Usage***
Andres Mariano Alonso and Luis Diambra

622 ***An Overview of the Temporal Shedding of SARS-CoV-2 RNA in Clinical Specimens***
Khrystyna Zhurakivska, Giuseppe Troiano, Giuseppe Pannone, Vito Carlo Alberto Caponio and Lorenzo Lo Muzio

631 ***COVID-19, Authoritarian Neoliberalism, and Precarious Migrant Work in Singapore: Structural Violence and Communicative Inequality***
Mohan Jyoti Dutta

649 ***Overview of the First 6 Months of Clinical Trials for COVID-19 Pharmacotherapy: The Most Studied Drugs***
Maria Laura Idda, Dorian Soru and Matteo Floris

656 ***Lung Mechanics of Mechanically Ventilated Patients With COVID-19: Analytics With High-Granularity Ventilator Waveform Data***
Huiqing Ge, Qing Pan, Yong Zhou, Peifeng Xu, Lingwei Zhang, Junli Zhang, Jun Yi, Changming Yang, Yuhan Zhou, Limin Liu and Zhongheng Zhang

665 ***COVID-19 Infection Among Healthcare Workers: Serological Findings Supporting Routine Testing***
Ariel D. Stock, Edward R. Bader, Phillip Cezayirli, Julio Inocencio, Samantha A. Chalmers, Reza Yassari, Vijay Yanamadala and Emad Eskandar

672 ***Efficacy of Nationwide Curfew to Encounter Spread of COVID-19: A Case From Jordan***
Moawiah Khatatbeh

675 ***Ozone Therapy as a Possible Option in COVID-19 Management***
Alessandra Gavazza, Andrea Marchegiani, Giacomo Rossi, Marianno Franzini, Andrea Spaterna, Sara Mangiaterra and Matteo Cerquetella

678 ***Repurposing Fragile X Drugs to Inhibit SARS-CoV-2 Viral Reproduction***
Cara J. Westmark, Maki Kiso, Peter Halfmann, Pamela R. Westmark and Yoshihiro Kawaoka

688 ***The Immune Response and Immunopathology of COVID-19***
Esmaeil Mortaz, Payam Tabarsi, Mohammad Varahram, Gert Folkerts and Ian M. Adcock

697 ***Germ-Free Mice Under Two-Layer Textiles Are Fully Protected From Bacteria in Sprayed Microdroplets: A Functional* in vivo *Test Method of Facemask/Filtration Materials***
Alex Rodriguez-Palacios, Mathew Conger and Fabio Cominelli

707 ***Unraveling the Epidemiology, Geographical Distribution, and Genomic Evolution of Potentially Lethal Coronaviruses (SARS, MERS, and SARS CoV-2)***
Nosheen Masood, Saima Shakil Malik, Muhammad Naqqi Raja, Sumaira Mubarik and Chuanhua Yu

715 ***Importance of Dietary Changes During the Coronavirus Pandemic: How to Upgrade Your Immune Response***
Ali Chaari, Ghizlane Bendriss, Dalia Zakaria and Clare McVeigh

739 ***Incidence and Persistence of Viral Shedding in COVID-19 Post-acute Patients With Negativized Pharyngeal Swab: A Systematic Review***
Giovanni Morone, Angela Palomba, Marco Iosa, Teodorico Caporaso, Domenico De Angelis, Vincenzo Venturiero, Anna Savo, Paola Coiro, Dario Carbone, Francesca Gimigliano, Giovanni Iolascon and Stefano Paolucci

748 ***What's Sex Got to Do With COVID-19? Gender-Based Differences in the Host Immune Response to Coronaviruses***
Nirupa Gadi, Samantha C. Wu, Allison P. Spihlman and Vaishali R. Moulton

770 ***The Metabolic Changes and Immune Profiles in Patients With COVID-19***
Bing He, Jun Wang, Yudie Wang, Juan Zhao, Juan Huang, Yu Tian, Cheng Yang, Heng Zhang, Mingxia Zhang, Lixing Gu, Xiaocui Zhou and Jingjiao Zhou

780 ***Research Collaboration and Outcome Measures of Interventional Clinical Trial Protocols for COVID-19 in China***
Ya Gao, Kelu Yang, Ming Liu, Yamin Chen, Shuzhen Shi, Fengwen Yang and Jinhui Tian

790 ***The Rapid Coronavirus Antibody Test: Can We Improve Accuracy?***
Ina P. Pavlova, Sujit S. Nair, Natasha Kyprianou and Ash K. Tewari

795 ***Scientific Rationale for a Bottom-Up Approach to Target the Host Response in Order to Try and Reduce the Numbers Presenting With Adult Respiratory Distress Syndrome Associated With COVID-19. Is There a Role for Statins and COX-2 Inhibitors in the Prevention and Early Treatment of the Disease?***
Geoffrey Mark Verrall

800 ***What Can We Estimate From Fatality and Infectious Case Data Using the Susceptible-Infected-Removed (SIR) Model? A Case Study of Covid-19 Pandemic***
Semra Ahmetolan, Ayse Humeyra Bilge, Ali Demirci, Ayse Peker-Dobie and Onder Ergonul

812 ***Anticipating the Novel Coronavirus Disease (COVID-19) Pandemic***
Taranjot Kaur, Sukanta Sarkar, Sourangsu Chowdhury, Sudipta Kumar Sinha, Mohit Kumar Jolly and Partha Sharathi Dutta

824 ***A Citizen Science Facemask Experiment and Educational Modules to Improve Coronavirus Safety in Communities and Schools***
Sarah E. Eichler, Austin P. Hopperton, Juan José Alava, Antonio Pereira Jr., Rukhsana Ahmed, Zisis Kozlakidis, Sanja Ilic and Alexander Rodriguez-Palacios

831 ***Clinical Characteristics and Outcomes of Severe and Critical Patients With 2019 Novel Coronavirus Disease (COVID-19) in Wenzhou: A Retrospective Study***
Song-Zan Qian, Wan-dong Hong, Lingjie-mao, Chenfeng-lin, Zhendong-fang and Jing-Ye Pan

838 ***Iran's Approach to COVID-19: Evolving Treatment Protocols and Ongoing Clinical Trials***
Ramin Rahmanzade, Reza Rahmanzadeh, Seyed MohammadReza Hashemian and Payam Tabarsi

846 ***The Relationship Between Chest Imaging Findings and the Viral Load of COVID-19***
Wei Zhao, Lei He, Haoneng Tang, Xingzhi Xie, Lingli Tang and Jun Liu

854 ***Characteristic of 523 COVID-19 in Henan Province and a Death Prediction Model***
Xiaoxu Ma, Ang Li, Mengfan Jiao, Qingmiao Shi, Xiaocai An, Yonghai Feng, Lihua Xing, Hongxia Liang, Jiajun Chen, Huiling Li, Juan Li, Zhigang Ren, Ranran Sun, Guangying Cui, Yongjian Zhou, Ming Cheng, Pengfei Jiao, Yu Wang, Jiyuan Xing, Shen Shen, Qingxian Zhang, Aiguo Xu and Zujiang Yu

867 ***The Impact of SARS-CoV-2 on the Most Common Comorbidities–A Retrospective Study on 814 COVID-19 Deaths in Romania***
Madalina Gabriela Barbu, Richard James Thompson, Dana Claudia Thompson, Dragos Cretoiu and Nicolae Suciu

882 ***Thoughts From the Trenches: Should We Look at the "Healthy"?***
Víctor M. Martínez-Taboada, Marcos López-Hoyos, Javier Crespo, Pedro Muñoz Cacho and José L. Hernández

886 ***A Basic Review of the Preliminary Evidence That COVID-19 Risk and Severity Is Increased in Vitamin D Deficiency***
Linda L. Benskin

911 ***Comparative Review of SARS-CoV-2, SARS-CoV, MERS-CoV, and Influenza A Respiratory Viruses***
Zeinab Abdelrahman, Mengyuan Li and Xiaosheng Wang

925 ***The Epidemiology of COVID-19 in the Gansu and Jinlin Provinces, China***
Jingchun Fan, Brett D. Hambly and Shisan Bao

932 ***Science and the War on Truth and Coronavirus***
Geoffrey P. Dobson

935 ***Seroprevalence of SARS-CoV-2 Among Pediatric Healthcare Workers in Spain***
Ana Dacosta-Urbieta, Irene Rivero-Calle, Jacobo Pardo-Seco, Lorenzo Redondo-Collazo, Antonio Salas, Jose Gómez-Rial and Federico Martinón-Torres

938 ***Seasonality of Respiratory Viral Infections: Will COVID-19 Follow Suit?***
Amani Audi, Malak AlIbrahim, Malak Kaddoura, Ghina Hijazi, Hadi M. Yassine and Hassan Zaraket

946 ***Analysis of Risk Factors for 24 Patients With COVID-19 Developing From Moderate to Severe Condition***
Dianming Li, Chuanmiao Liu, Jiahui Liu, Junfeng Hu, Yanli Yang and Yufu Zhou

953 ***Epidemiological Chronicle of the First Recovered Coronavirus Disease Patient From Panama: Evidence of Early Cluster Transmission in a High School of Panama City***
Augusto Hernandez, Paul Muñoz, Jose C. Rojas, Gilberto A. Eskildsen, Julio Sandoval, K. S. Rao, Rolando A. Gittens and Jose R. Loaiza

960 ***Early COVID-19 Interventions Failed to Replicate 1918 St. Louis vs. Philadelphia Outcomes in the United States***
Aliea M. Jalali, Brent M. Peterson and Thushara Galbadage

968 ***Therapeutic Options Against the New Coronavirus: Updated Clinical and Laboratory Evidences***
Amélia Carolina Lopes Fernandes, Adson José Martins Vale, Fausto Pierdoná Guzen, Francisco Irochima Pinheiro, Ricardo Ney Cobucci and Eduardo Pereira de Azevedo

990 ***Insights on SARS-CoV-2 Molecular Interactions With the Renin-Angiotensin System***
Larissa Braga Costa, Lucas Giandoni Perez, Vitória Andrade Palmeira, Thiago Macedo e Cordeiro, Victor Teatini Ribeiro, Katharina Lanza and Ana Cristina Simões e Silva

1003 ***Prevention and Control of COVID-19 in Italian Prisons: Stringent Measures and Unintended Consequences***
Lara Tavoschi, Roberto Monarca, Ruggero Giuliani, Alessio Saponaro, Stefano Petrella, Roberto Ranieri, Filipa Alves da Costa, Carina Ferreira-Borges and Linda Montanari

1006 ***Flexible, Functional, and Familiar: Characteristics of SARS-CoV-2 Spike Protein Evolution***
Dianita S. Saputri, Songling Li, Floris J. van Eerden, John Rozewicki, Zichang Xu, Hendra S. Ismanto, Ana Davila, Shunsuke Teraguchi, Kazutaka Katoh and Daron M. Standley

1012 ***Direct Clinical Evidence Recommending the Use of Proteinase K or Dithiothreitol to Pretreat Sputum for Detection of SARS-CoV-2***
Jing Peng, Yanjun Lu, Juan Song, Bruce A. Vallance, Kevan Jacobson, Hong Bing Yu and Ziyong Sun

1017 ***A Pattern Categorization of CT Findings to Predict Outcome of COVID-19 Pneumonia***
Chao Jin, Cong Tian, Yan Wang, Carol C. Wu, Huifang Zhao, Ting Liang, Zhe Liu, Zhijie Jian, Runqing Li, Zekun Wang, Fen Li, Jie Zhou, Shubo Cai, Yang Liu, Hao Li, Zhongyi Li, Yukun Liang, Heping Zhou, Xibin Wang, Zhuanqin Ren and Jian Yang

1031 ***Strategies for Targeting SARS CoV-2: Small Molecule Inhibitors—The Current Status***
Narasimha M. Beeraka, Surya P. Sadhu, SubbaRao V. Madhunapantula, Rajeswara Rao Pragada, Andrey A. Svistunov, Vladimir N. Nikolenko, Liudmila M. Mikhaleva and Gjumrakch Aliev

1053 ***Antibody Profiling of COVID-19 Patients in an Urban Low-Incidence Region in Northern Germany***
Werner Solbach, Julia Schiffner, Insa Backhaus, David Burger, Ralf Staiger, Bettina Tiemer, Andreas Bobrowski, Timothy Hutchings and Alexander Mischnik

1061 ***The CHIR Score for Evaluating the Hyperimmune Response in COVID-19: A Preliminary Concept***
Daniel Kumar Goyal and Fatma Mansab

1065 ***Geographical Distribution of Genetic Variants and Lineages of SARS-CoV-2 in Chile***
Andrés E. Castillo, Bárbara Parra, Paz Tapia, Jaime Lagos, Loredana Arata, Alejandra Acevedo, Winston Andrade, Gabriel Leal, Carolina Tambley, Patricia Bustos, Rodrigo Fasce and Jorge Fernández

1072 ***Meningitis as an Initial Presentation of COVID-19: A Case Report***
Sidra Naz, Muhammad Hanif, Muhammad Adnan Haider, Mukarram Jamat Ali, Muhammad Umer Ahmed and Sana Saleem

1076 ***Increased Serum Levels of sCD14 and sCD163 Indicate a Preponderant Role for Monocytes in COVID-19 Immunopathology***
Jose Gómez-Rial, Maria José Currás-Tuala, Irene Rivero-Calle, Alberto Gómez-Carballa, Miriam Cebey-López, Carmen Rodríguez-Tenreiro, Ana Dacosta-Urbieta, Carmen Rivero-Velasco, Nuria Rodríguez-Núñez, Rocio Trastoy-Pena, Javier Rodríguez-García, Antonio Salas and Federico Martinón-Torres

1084 ***The Possible Dual Role of the ACE2 Receptor in Asthma and Coronavirus (SARS-CoV2) Infection***
Anna Cláudia Calvielli Castelo Branco, Maria Notomi Sato and Ricardo Wesley Alberca

1090 ***How to Understand "Herd Immunity" in COVID-19 Pandemic***

Yuanqing Xia, Lumin Zhong, Jingcong Tan, Zhiruo Zhang, Jiajun Lyu, Yiting Chen, Anda Zhao, Lili Huang, Zichong Long, Ning-Ning Liu, Hui Wang and Shenghui Li

1097 ***The Pipeline of Therapeutics Testing During the Emergency Phase of the COVID-19 Outbreak***

Marco Canevelli, Giulia Remoli, Federica Trentin, Gabriele Riccardi, Leonardo Tariciotti, Giovanni Risoleo, Antonio Ancidoni, Giuseppe Bruno, Matteo Cesari, Nicola Vanacore and Valeria Raparelli

1108 ***COMOKIT: A Modeling Kit to Understand, Analyze, and Compare the Impacts of Mitigation Policies Against the COVID-19 Epidemic at the Scale of a City***

Benoit Gaudou, Nghi Quang Huynh, Damien Philippon, Arthur Brugière, Kevin Chapuis, Patrick Taillandier, Pierre Larmande and Alexis Drogoul

frontiers in Medicine

SYSTEMATIC REVIEW
published: 21 July 2020
doi: 10.3389/fmed.2020.00459

COVID-19 Clinical Characteristics, and Sex-Specific Risk of Mortality: Systematic Review and Meta-Analysis

Mohammad Javad Nasiri[1‡], Sara Haddadi[2‡], Azin Tahvildari[3†], Yeganeh Farsi[3†], Mahta Arbabi[3†], Saba Hasanzadeh[3†], Parnian Jamshidi[3†], Mukunthan Murthi[2] and Mehdi Mirsaeidi[2,4]*

[1] Department of Microbiology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran, [2] Division of Pulmonary and Critical Care, Department of Medicine, University of Miami Miller School of Medicine, Miami, FL, United States, [3] Student Research Committee, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran, [4] Department of Pulmonary and Critical Care, Miami VA Medical Center, Miami, FL, United States

OPEN ACCESS

Edited by:
Zisis Kozlakidis, International Agency for Research on Cancer (IARC), France

Reviewed by:
Ricvan Dana Nindrea, Andalas University, Indonesia
Hui Wang, Shanghai Jiao Tong University, China

***Correspondence:**
Mehdi Mirsaeidi
msm249@med.miami.edu

[†] These authors have contributed equally to this work

[‡] These authors share first authorship

Specialty section:
This article was submitted to Infectious Diseases, Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: 03 April 2020
Accepted: 09 July 2020
Published: 21 July 2020

Citation:
Nasiri MJ, Haddadi S, Tahvildari A, Farsi Y, Arbabi M, Hasanzadeh S, Jamshidi P, Murthi M and Mirsaeidi M (2020) COVID-19 Clinical Characteristics, and Sex-Specific Risk of Mortality: Systematic Review and Meta-Analysis. Front. Med. 7:459. doi: 10.3389/fmed.2020.00459

Background: The rapidly evolving coronavirus disease 2019 (COVID-19), was declared a pandemic by the World Health Organization on March 11, 2020. It was first detected in the Wuhan city of China and has spread globally resulting in a substantial health and economic crisis in many countries. Observational studies have partially identified different aspects of this disease. There have been no published systematic reviews that combine clinical, laboratory, epidemiologic, and mortality findings. Also, the effect of gender on the outcomes of COVID-19 has not been well-defined.

Methods: We reviewed the scientific literature published from January 1, 2019 to May 29, 2020. Statistical analyses were performed with STATA (version 14, IC; Stata Corporation, College Station, TX, USA). The pooled frequency with 95% confidence intervals (CI) was assessed using random effect model. $P < 0.05$ was considered a statistically significant publication bias.

Results: Out of 1,223 studies, 34 satisfied the inclusion criteria. A total of 5,057 patients with a mean age of 49 years were evaluated. Fever (83.0%, CI 77.5–87.6) and cough (65.2%, CI 58.6–71.2) were the most common symptoms. The most prevalent comorbidities were hypertension (18.5%, CI 12.7–24.4) and Cardiovascular disease (14.9%, CI 6.0–23.8). Among the laboratory abnormalities, elevated C-Reactive Protein (CRP) (72.0%, CI 54.3–84.6) and lymphopenia (50.1%, CI 38.0–62.4) were the most common. Bilateral ground-glass opacities (66.0%, CI 51.1–78.0) was the most common CT scan presentation. The pooled mortality rate was 6.6%, with males having significantly higher mortality compared to females (OR 3.4; 95% CI 1.2–9.1, $P = 0.01$).

Conclusion: COVID-19 has caused a significant number of hospitalization and mortality worldwide. Mortality associated with COVID-19 was higher in our study compared to the previous reports from China. The mortality was significantly higher among the hospitalized male group. Further studies are required to evaluate the effect of different variables resulting in sex disparity in COVID-19 mortality.

Keywords: coronavirus, COVID-19, mortality, male, pandemic

INTRODUCTION

Facing an immediate crisis by the novel coronavirus, Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) which has been called the once in a century pathogen, requires a global response (1). The disease caused by this virus has been named "coronavirus disease 2019" (COVID-19) by the World Health Organization (WHO). As of now, more than 180 countries have reported COVID-19 patients. Given the increasing number of countries infected with SARS-CoV-2, WHO finally classified COVID-19 as a pandemic on March 11, 2020 (2). The SARS-CoV-2 virus is a beta-coronavirus, belonging to the same coronavirus family as the Middle East Respiratory Syndrome virus (MERS-CoV) and SARS-CoV. MERS-CoV and SARS-CoV were previously responsible for respiratory syndrome outbreaks. However, COVID-19 is the first virus of the coronavirus family to cause a pandemic (3).

COVID-19 started in China in December 2019 when a cluster of patients with pneumonia of unknown origin were identified in the city of Wuhan. Since then, it has infected hundreds of thousands of people around the world and resulted in more than 539,900 deaths up to this date (4). Despite governmental travel restrictions in many countries, the confirmed number of new cases has been rising globally. The international community has asked for at least 675 million US dollars to use for preparedness and protection of states with weaker health systems (5).

In the previous two outbreaks of coronaviral respiratory illness, namely Severe Acute Respiratory Illness (SARS) and Middle East Respiratory Illness (MERS), gender-based differences in mortality were observed. In SARS, mortality risk was twice as high in younger males compared to younger females, but this difference in mortality decreased with older age. Additionally, the case fatality rate observed in males was twice that of females in MERS (6). The effect of sex on COVID-19 mortality was unknown. In our systematic review, we compared male and female mortality risk for COVID-19.

The novelty of COVID-19 has raised many questions about the epidemiology of the disease, clinical and laboratory methods of diagnosis, as well as therapeutic measures. Many observational studies have been dealing with these features separately. Further combined systematic reviews are needed, to understand the role of sex in COVID-19 associated mortality. In this meta-analysis study, we reviewed the published literature from January 1, 2019 to May 29, 2020 to provide a comprehensive overview of COVID-19.

METHODS

Search Strategy

We searched Pubmed/Medline, Embase, Web of Science, and the Cochrane Library for studies published from January 1, 2019 to May 29, 2020. The search strategy was based on the following key-words: COVID-19, severe acute respiratory syndrome coronavirus 2, novel coronavirus, SARS-CoV-2, nCoV disease, SARS2, COVID19, Wuhan coronavirus, Wuhan seafood market pneumonia virus, 2019-nCoV, coronavirus disease-19, coronavirus disease 2019, 2019 novel coronavirus and Wuhan pneumonia. Lists of references of selected articles and relevant review articles were hand-searched to identify further studies. This study was conducted and reported according to the PRISMA guidelines (7). The study did not require Institutional Review Board approval.

Study Selection

The records found through database searching were merged and the duplicates were removed using EndNote X7 (Thomson Reuters, New York, NY, USA). Two reviewers (YF and PJ) independently screened the records by title and abstract to exclude those not related to the current study. The full texts of potentially eligible records were retrieved and evaluated by a third reviewer (AT). Included studies met the following inclusion criteria: (i) patients were confirmed and diagnosed with RT-PCR as suggested by WHO; (ii) The raw data for clinical, radiological and laboratory findings were included; and (iii) the outcomes were addressed. Studies with insufficient information about patients' characteristics and outcomes were excluded. Case reports, reviews, and animal studies were also excluded. Only studies written in English were selected.

Data Extraction and Quality Assessment

A data extraction form was designed by two reviewers (AZ and SH). These reviewers extracted the data from all eligible studies and differences were resolved by consensus. The following data was extracted: first author name; year of publication; type of study; country(ies) where the study was conducted; distribution of age and sex in the population; number of patients investigated; data for clinical, radiological, and laboratory findings; and outcomes.

Data Synthesis and Analysis

Statistical analyses were performed with STATA (version 14, IC; Stata Corporation, College Station, TX, USA). The pooled frequency with 95% confidence intervals (CI) was assessed using random effect model. The between-study heterogeneity was assessed by Cochran's Q and the I2 statistic. Publication bias was assessed statistically by using Begg's and Egger's tests ($p < 0.05$ was considered indicative of statistically significant publication bias).

Quality Assessment

The checklist provided by the Joanna Briggs Institute (JBI) was used to perform quality assessment (8).

RESULTS

The search yielded 1,223 publications, of which 280 potentially eligible studies were identified for full-text review, resulting in 34 studies fulfilling the inclusion criteria (**Figure 1**) (**Table 1**). A total of 5,057 patients were included, of which the mean age was 49.0 years. Based on JBI tool, the included studies had a low risk of bias.

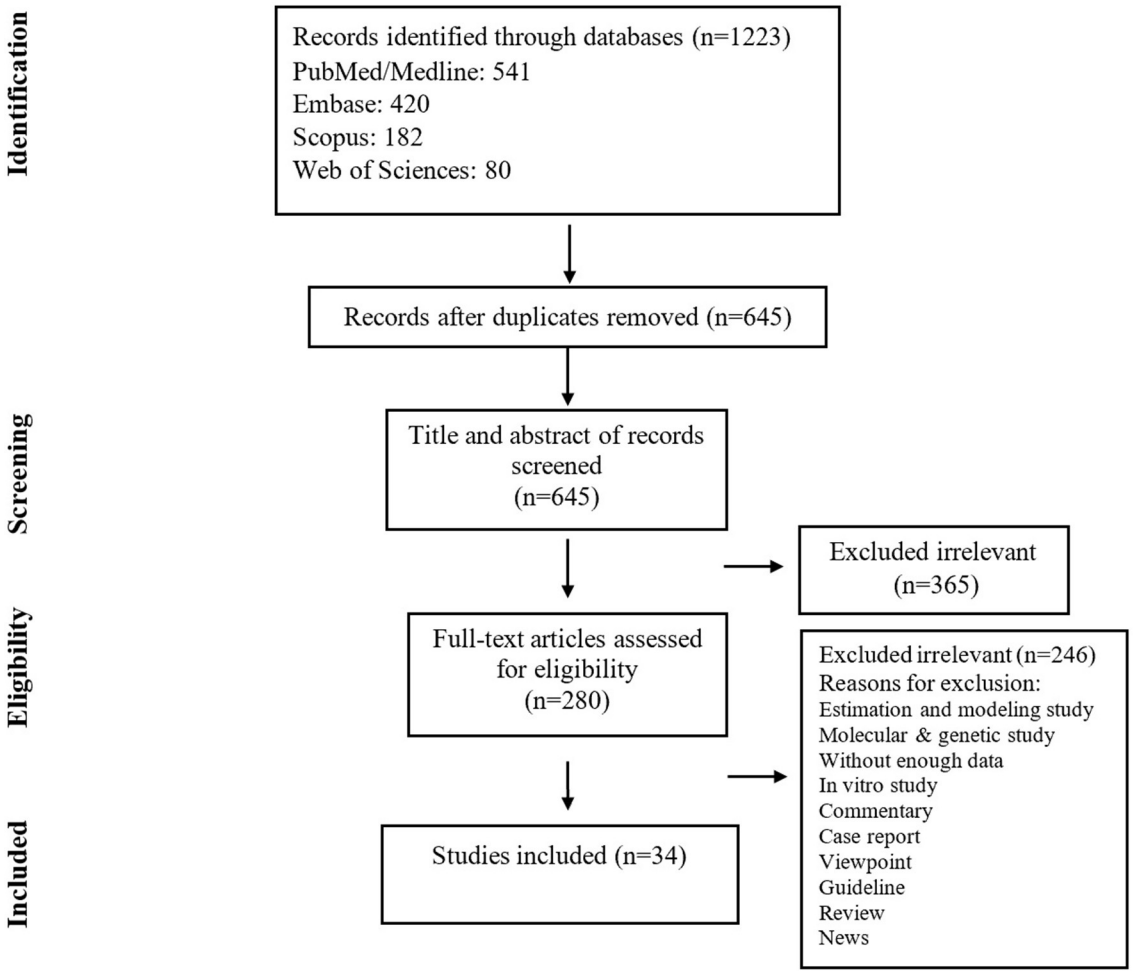


FIGURE 1 | Flow chart of study selection for inclusion in the systematic review and meta-analysis.

Clinical Manifestations and Comorbidities

The most common signs and symptoms were fever (83.0%, CI 77.5–87.6), cough (65.2%, CI 58.6–71.2), dyspnea (27.4%, CI 19.6–35.2), myalgia/fatigue (34.7%, CI 26.0–44.4), and Sputum production (17.2%, CI 10.8–26.4). Less common symptoms included hemoptysis (2.4%, CI 0.8–6.7), diarrhea (5.7%, CI 3.8–8.6), and nausea/vomiting (5.0%, CI 2.3–10.7) (**Table 2**).

The most common comorbidities were hypertension (18.5%, CI 12.7–24.4), cardiovascular diseases (14.9%, CI 6.0–23.8), diabetes (10.8%, CI 8.3–13.3), chronic liver disease (8.1, CI 4.6–11.6) and smoking (8.0%, CI 2.3–13.6), respectively (**Table 3**).

Lab Abnormalities and Complications

The most frequent abnormal laboratory findings in patients with COVID-19 were, respectively, elevated C-Reactive Protein (CRP) (72% CI 54.3–84.6), lymphopenia (50.1%, CI 38.0–62.4), elevated Lactate Dehydrogenase (LDH) (41%, CI 22.8–62.0), elevated serum aspartate aminotransferase (19.7%, CI 10.5–33.7), and thrombocytopenia (11.1%, CI 7.7–15.7) (**Table 4**). Among the confirmed COVID-19 subjects, 14.0% (CI, 6.7–29.0) had viremia. Impaired hepatic function with ALT levels >47.25 U/L was seen in 13.3% (CI 3.2–41.0) of COVID-19 subjects. Acute cardiac injury with troponin levels >28 pg/ml was seen in 12.4% (CI 6.2–23.2). Acute kidney injury was found in 5.5% (CI 1.3–20.8). Shock was reported in 4.0% (CI 1.6–12.0). Finally, 13.0% (CI 4.8–30.0) met the definition of acute respiratory distress syndrome (ARDS).

Radiological Characteristics

Chest X-Ray (CXR) and Chest CT scan were the most common imaging modalities used for the diagnosis of COVID-19. The pooled sensitivity of CT scan for detecting COVID-19 was 79.3%. The most common sites of the lung involvement based on chest CT scan were right lower lobe (76.2%, CI 57.8–82.5) followed by the left lower lobe (71.8%, CI 57.8–82.5). Most of the patients (74.8%) had bilateral involvement. The most common pattern of parenchymal involvement was ground-glass opacities (66.0%, CI 51.1–78.0). The Chest CT scan was reported normal in 20.7% of the patients with confirmed RT-PCR results (**Table 5**).

TABLE 1 | Characteristics of the included studies.

First author	Country	Published time	Type of study	Mean age	Male/female	Nationality	No. of patients	Diagnostic methods
Hui et al. (9)	China	14, Jan, 2020	Case series	NR	NR	Chinese	41	RT-PCR/CT-scan
Xia et al. (10)	China	26, Feb, 2020	Case series	54.5	21M, 9F	Chinese	30	RT-PCR
Xu et al. (11)	China	13, Feb, 2020	Case series	41	M35, F27	Chinese	62	RT-PCR
Zhang et al. (12)	China	7, Feb, 2020	Case series	NR	NR	Chinese	178	RT-PCR
To et al. (13)	China	12, Feb, 2020	Case series	62.5	7M, 5F	Chinese	12	RT-PCR
Zou et al. (14)	China	19, Feb, 2020	Correspondence	59	9M,9F	Chinese	18	RT-PCR
Hoehl et al. (15)	Germany	3, Mar, 2020	Correspondence	35	NR	German	126	RT-PCR/CT-scan
Pan et al. (16)	China	24, Feb, 2020	Correspondence	NR	NR	Chinese	82	RT-PCR/CT-scan
Tang et al. (17)	China	19, Feb, 2020	Cross-sectional	54	98M, 85F	Chinese	183	RT-PCR
Chung et al. (18)	China	4, Feb, 2020	Cross-sectional	51	M13, F8	Chinese	21	RT-PCR/CT-scan
Fang et al. (19)	China	19, Feb, 2020	Cross-sectional	45	29M, 22F	Chinese	51	RT-PCR/CT-scan
Guan et al. (20)	China	28, Feb, 2020	Cross-sectional	47	640M,459F	Chinese	1099	RT-PCR/CT-scan
Huang et al. (21)	China	24, Jan, 2020	Cross-sectional	49	30M,11F	Chinese	41	RT-PCR
Kui et al. (22)	China	7, Feb, 2020	Cross-sectional	57	61M,76F	Chinese	137	RT-PCR
Li et al. (23)	China	29, Jan, 2020	Cross-sectional	52	M238, F187	Chinese	425	RT-PCR/CT-scan
Liu et al. (24)	China	9, Feb, 2020	Cross-sectional	53.6	8M, 4F	Chinese	12	RT-PCR/CT-scan
Wang et al. (25)	China	7, Feb, 2020	Cross-sectional	56	75M, 63F	Chinese	138	RT-PCR/CT-scan
Wu et al. (26)	China	29, Feb, 2020	Cross-sectional	46	39M, 41F	Chinese	80	RT-PCR
Zhang et al. (27)	China	19, Feb, 2020	Cross-sectional	57	71M,69F	Chinese	140	RT-PCR
Ai et al. (28)	China	26, Feb, 2020	Cross-sectional	48.5	M467, F547	Chinese	1014	RT-PCR/CT scan
Pan et al. (29)	China	13, Feb, 2020	Cross-sectional	40	6M, 15F	Chinese	21	RT-PCR/CT-scan
Shi et al. (30)	China	24, Feb, 2020	Cross-sectional	49.5	42M, 39F	Chinese	81	RT-PCR/CT-scan
Yang et al. (31)	China	21, Feb, 2020	Cross-sectional	59.7	35M, 17F	Chinese	52	RT-PCR
Bajema et al. (32)	China	4, Feb, 2020	Cross-sectional	NR	115M, 95F	Chinese	210	RT-PCR/CT-scan
Bernheim et al. (33)	China	20, Feb, 2020	Cross-sectional	45.3	61M, 60F	Chinese	121	RT-PCR
Chen et al. (34)	China	15, Feb, 2020	Cross-sectional	55.5	67M, 32F	Chinese	99	RT-PCR
Pan et al. (35)	China	13, Feb, 2020	Cross-sectional	45	33M, 30F	Chinese	63	RT-PCR
Xu et al. (36)	China	21, Feb, 2020	Cross-sectional	44	29M, 21F	Chinese	50	RT-PCR/CT-scan
Xu et al. (37)	China	28, Feb, 2020	Cross-sectional	50	39M, 51F	Chinese	90	RT-PCR
Chang et al. (38)	China	7, Feb, 2020	Research letter	34	10M, 3F	Chinese	13	RT-PCR/CT-scan
Chen et al. (39)	China	26, Feb, 2020	Research letter	NR	NR	Chinese	85	RT-PCR/CT-scan
Kwok et al. (40)	China	7, Feb, 2020	Research letter	59.8	9M, 5F	Chinese	14	RT-PCR/CT-scan
Hansen et al. (41)	Norway	23 April, 2020	Cross-sectional	72.5	28M,14F	Norwegian	42	RT-PCR/CT-scan
Yu et al. (42)	China	14, May, 2020	Cross-sectional	64	139 M, 87 F	Chinese	226	RT-PCR/CT-scan

TABLE 2 | Meta-analysis of comorbidities.

	Pooled frequency	*n*/*N**	Publication bias	Heterogeneity test	
	(*p*-value)		(*p*-value)	I^2 (%)	*p* value
Smoking	8.0 (2.3–13.6)	172/1,332	0.06	100	0.00
Hypertension	18.5 (12.7–24.4)	306/1,800	0.98	100	0.00
Cardiovascular disease	14.9 (6.0–23.8)	178/2,031	0.72	100	0.00
Diabetes	10.8 (8.3–13.3)	166/1,932	0.39	100	0.00
Pulmonary disease	3.4 (0.8–6.0)	39/2,031	0.72	100	0.00
Malignancies	2.8 (0.8–4.8)	33/1,816	0.74	100	0.00
Chronic liver disease	8.1 (4.6–11.6)	29/546	0.45	100	0.00
Renal disease	4.4 (0.24–8.6)	17/1,472	0.33	100	0.00

**n, number of patients with comorbidity; N, total number of patients.*

TABLE 3 | Meta-analysis of clinical manifestations.

	Pooled frequency (95% CI)	*n*/*N**	Publication bias (*p*-value)	Heterogeneity test	
				I^2 (%)	*p*-value
Fever	83.0 (77.5–87.6)	2,073/2,465	0.76	86	0.00
Cough	65.2 (58.6–71.2)	1,689/2,515	0.80	85	0.00
Dyspnea	27.4 (19.6–35.2)	477/2,014	0.42	89	0.00
Myalgia/fatigue	34.7 (26.0–44.4)	742/1,938	0.60	89	0.00
Sputum production	17.2 (10.8–26.4)	480/1,862	0.01	89	0.00
Sore throat	14.5 (10.6–19.5)	224/1,577	0.88	66	0.00
Headache	11.1 (7.7–15.7)	230/1,864	0.30	74	0.00
Diarrhea	5.7 (3.8–8.6)	104/2,041	0.77	66	0.00
Hemoptysis	2.4 (0.8–6.7)	20/1,339	0.77	100	0.00
Anorexia	10.1 (1.0–57.2)	82/1,322	0.73	98	0.00
Nausea/vomiting	5.0 (2.3–10.7)	65/1,563	0.90	85	0.00
Dizziness	8.6 (2.5–26.0)	16/205	0.90	65	0.00
Chest tightness	8.4 (2.5–26.0)	24/256	0.24	78	0.00
Rhinorrhea	9.3 (2.2–31.0)	28/232	0.17	88	0.00
Chills	14.3 (3.0–47.4)	12/111	NA	86	0.00

TABLE 4 | Meta-analysis of laboratory findings.

	Pooled frequency (95% CI)	*n*/*N**	Publication bias (*p*-value)	Heterogeneity test	
				I^2 (%)	*p*-value
Lymphopenia	50.1 (38.0–62.4)	1,122/1,853	0.08	93	0.00
Lymphocytosis	33.5 (2.4–90.2)	55/93	NA	88	0.00
Neutrophilia	29.7 (19.3–42.7)	60/191	0.51	58.7	0.08
Leukopenia	28.0 (20.0–37.4)	544/1,798	0.89	88	0.00
Leukocytosis	10.8 (5.8–19.1)	165/1,829	0.86	92	0.00
Thrombocytopenia	11.1 (7.7–15.7)	343/1,393	0.00	86	0.00
Anemia	43.5 (30.3–57.7)	79/179	NA	72	0.00
Decreased albumin	51.8 (2.0–98.0)	105/191	0.99	96	0.00
High CRP	72.0 (54.3–84.6)	918/1,681	0.02	96	0.00
High LDH	41.0 (22.8–62.0)	408/1,393	0.32	94	0.00
High ESR	79.7 (66.6–88.5)	143/179	NA	69	0.00
High AST	19.7 (10.5–33.7)	267/1,474	0.70	93	0.00
High ALT	14.6 (7.6–26.3)	191/1,290	0.99	84.8	0.00
High creatinine kinase	14.1 (8.3–23.0)	142/1,453	0.20	84	0.00
High bilirubin	7.9 (2.9–19.0)	95/1,278	0.96	89	0.00
High creatinine	3.3 (1.2–9.1)	20/1,294	0.13	74	0.00
High troponin I	2.4 (0.3–15.0)	1/41	NA	0.00	0.1

Outcomes

94.6% (CI 73.8–99.1) of the patients with severe COVID-19 were hospitalized. The pooled mortality rate of these patients was 6.6% (CI 2.8–15.0) (**Tables 6**, **7**). Old age, male sex, presence of underlying diseases, higher level of D-dimer, lower level of fibrinogen and anti-thrombin, progressive radiographic deterioration on follow up CT scans, development of ARDS, and requirement of mechanical ventilation were all reported factors associated with increased mortality rate. As shown in **Table 8**, men had significantly higher mortality in the hospital compared to women (OR 3.4; 95% CI 1.2–9.1, $P = 0.01$). Although ICU admission was higher in men, the difference was not statistically significant. The mean duration between the time of hospitalization and death was 17.5 days with minimum and maximum periods of 14 and 21 days, respectively. The effects and summaries calculated using a random-effects model weighted by the study population is shown in **Figure 2**.

DISCUSSION

We evaluated the signs and symptoms, diagnostic modalities, therapeutic measures, and epidemiologic features of COVID-19 to have a better understanding of this pandemic caused

TABLE 5 | Meta-analysis of imaging findings.

CT Scan	Patterns		Pooled frequency (95% CI)	*n/N**	Publication bias (*p*-value)	Heterogeneity test	
						I^2 (%)	*p*-value
Location of involvement	Number of affected lobe	Unaffected	20.7 (15.1–27.6)	33/161	0.18	0.0	0.57
		1 lobe	14.8 (7.4–24.0)	52/318	0.22	73	0.00
		2 lobes	9.5 (6.5–12.8)	30/318	0.32	0.0	0.50
		3 lobes	11.7 (7.9–14.6)	36/318	0.64	0.0	0.50
		4 lobes	15.8 (10.3–20.7)	49/318	0.90	40	0.15
		5 lobes	37.2 (32.0–42.3)	118/318	0.50	30	0.22
	Affected lobe (s)	RUL	56.8 (50.6–62.8)	145/255	0.12	52	0.10
		RML	48.6 (42.5–54.8)	124/255	0.07	0.0	0.48
		RLL	76.2 (65.5–84.4)	193/255	0.14	64	0.03
		LUL	56.0 (47.1–64.7)	153/255	0.12	0.0	0.40
		LLL	71.8 (57.8–82.5)	167/234	0.30	76	0.01
	Laterality	Uni lateral	28.8 (16.6–45.2)	62/205	0.80	77	0.01
		Bi lateral	70.6 (55.3–82.5)	142/205	0.20	74	0.01
Pattern of involvement	Pattern of involvement	No involvement	17.2 (11.4–25.0)	193/1,080	0.42	63.0	0.04
		Both of GGO* & consolidation	39.0 (28.1–51.0)	57/142	NA	25	0.24
		GGO without consolidation	66.0 (51.1–78.0)	846/1,365	0.67	90	0.00
		Consolidation without GGO	9.4 (3.3–23.6)	26/274	0.21	82	0.00
	Laterality	Uni lateral	21.8 (12.0–36.3)	101/507	0.63	87	0.00
		Bi lateral	74.8 (62.5–84.0)	405/548	0.29	84	0.00

GGO, Ground Glass Opacities.

TABLE 6 | Meta-analysis of complications.

	Pooled frequency (95% CI)	*n/N**	Publication bias (*p*-value)	Heterogeneity test	
				I^2 (%)	*p*-value
RNAemia	14.0 (6.7–29.0)	6/41	NA	0.00	1.00
ARDS	13.0 (4.8–30.0)	142/1,794	0.67	96	0.00
Acute cardiac injury	12.4 (6.2–23.2)	28/243	0.83	65	0.03
Acute kidney injury	5.5 (1.3–20.8)	34/1,441	0.58	93	0.00
Liver failure	13.3 (3.2–41.0)	20/144	0.50	84	0.00
Shock	4.0 (1.6–12.0)	32/1,389	0.60	86	0.00
Hospitalization	94.6 (73.8–99.1)	1,561/1,829	0.76	98	0.00

TABLE 7 | Meta-analysis of outcomes.

	Pooled frequency (95% CI)	*n/N**	Publication bias (*p*-value)	Heterogeneity test	
				I^2 (%)	*p*-value
Discharged	52.7 (36.5–68.4)	486/948	0.44	93	0.00
Death	6.6 (2.8–15.0)	111/2,026	0.50	93	0.00

by SARS-CoV-2. The pooled mortality rate of these patients was 6.6% overall. We detected several factors that contributed to a worsened outcome including old age, male sex, presence of underlying diseases, and abnormal laboratory finding such as an elevated D-Dimer. Although there was not a significant difference between male and female gender in ICU admissions, male gender showed a significantly higher in-hospital mortality rate.

D-Dimer >1 μg/mL was identified as an associative factor that increased odds of in-hospital death in a study by Zhou et al. ($p = 0.0033$) (43).

Another significant finding in our analysis was the incidence of cardiac injury in 12.4% of the patients, which is a common event seen in a multitude of viral illnesses (44). Gao et al. observed that subjects with influenza (H7N9) and cardiac injury had an elevated risk of mortality (HR = 2.06) (45). In a study by Ludwig

TABLE 8 | Mortality and ICU admission in men vs. women in patients with COVID-19.

	Pooled OR (95% CI)	*p*-value	Heterogeneity test	
			I^2 (%)	*p*-value
Mortality in men vs. women	3.4 (1.2–9.1)	0.01	0.00	0.6
ICU admission in men vs. women	1.6 (0.7–3.2)	0.1	0.00	0.5

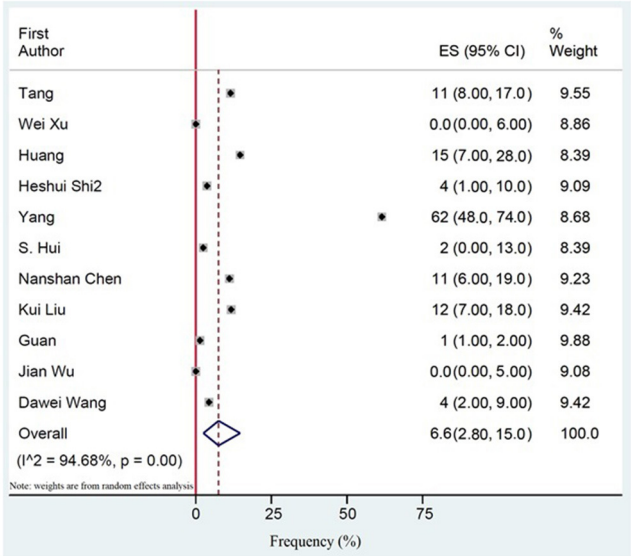


FIGURE 2 | The pooled mortality rate of patients with COVID-19. Effects and summaries were calculated using a random-effects model weighted by study population.

et al. which analyzed cardiac biomarkers in influenza patients, 24% of the subjects showed acute cardiac injury ≤30 days after influenza diagnosis and half of the injuries included myocardial infarctions (46). Although our analysis did not show increased mortality risk in patients with cardiac injury, these findings could indicate the potential need for identifying and optimizing cardiac risk factors in COVID-19 patients during the treatment period.

The mean duration between hospitalization and death was 17.5 days (range: 4–21 days), compared to 17.4 days in SARS (47). The overall mortality rate in this study was 6.6%, which is more than twice that was reported earlier (20). Though comparable mortality was reported by Li et al. (7%) and Qian et al. (8.9%) in their meta-analyses, a study by Rodriguez et al. showed a much higher death rate of 13.9% (48–50). On the other hand, a study from the Jiangsu province of China results showed a high cure rate equal to 96.67%. Although the main reason for very low mortality in this study remains unknown, measures including early recognition and centered-quarantine may be contributing factors (51).

Of note, the in-hospital mortality of males was significantly higher than that of females (OR 3.4; 95% CI 1.2–9.1, $P = 0.01$). A similar pattern of higher mortality in males has been reported in previous coronavirus outbreaks of SARS and MERS. Karlberg et al. also reported that the gender-based difference in mortality was higher in younger males (0–44 years) (RR = 2), compared to those of age group 45–74 (RR-1.45) (52). Similarly, the study by Alghamdi et al. showed that the case fatality rate in males was twice that of females in MERS (52 vs. 23%) (6). Although a gender-based difference in the immune response to infections has been suggested as a possible factor, other contributing factors including smoking history and severity of underlying comorbidities cannot be ruled out (53). This is especially of significance in China, where the prevalence of smoking among men (57.6%) is almost 10 times higher that of women (6.7%) (54). This difference in mortality opens the discussion for the need to treat COVID-19 more aggressively in males, including the possibility of earlier intubation and mechanical ventilation in this population. Cigarette Smokers showed to have a higher expression of Angiotensin converting enzyme 2 (ACE2) in lower airways. As it was discussed, ACE2 is the receptor for SARSCoV-2 in the lower respiratory tracts. This finding suggests that smokers are at a higher risk for COVID-19 (55). Therefore we emphasize on smoking cessation especially in the male group with COVID-19. Men smoke more than five times as much as women. (35% in males compared to 6% in females). Although this ratio varies in different countries, it is true that men smoke more in almost all countries (56). These findings can suggest part of the reason behind the significant higher mortality in males with COVID-19. Further investigations are needed to understand this phenomenon.

According to Xiaochen Li et al. male, elder age, leukocytosis, high LDH level, cardiac injury, hyperglycemia and chronic corticosteroid use were related to a higher risk of death in COVID-19. Male group counted for slightly more than half of all their patients (50.9%), however 56.9% of the severe COVID-19 cases were males compared to 45.2% females ($P = 0.006$). They showed that 19.2% of patients with severe COVID-19 were smokers (57).

Ruan et al. studied 68 deceased cases and 82 discharged ones to identify the clinical predictors of COVID-19 mortality, they found a significant difference among patients with Cardiovascular diseases ($p < 0.001$), however, their study didn't show any significant difference in sex ratio between the death group and the discharge group. ($P < 0.43$) (58).

Obesity is a risk factor for comorbid conditions such as cardiovascular diseases which are associated with a higher COVID-19 related deaths. Simonnet et al. showed that invasive mechanical ventilation was significantly associated with male sex ($p < 0.05$) and Body Mass Index (BMI) ($p < 0.05$), independent of age, diabetes, and hypertension (59). Previous studies had shown a low mortality rate in obese and morbid obese patients presenting with ARDS which is defined as obesity paradox. There is still more data required to identify whether this paradox is broken by COVID-19 (60).

According to Zirui Tay et al. there may be alleles on the location of *ACE2* on X-chromosome that confer resistance to COVID-19. This may explain the lower mortality among females. Additionally, estrogen and testosterone sex hormones

can modulate the immune response. Therefore, the disease severity may vary based on the hormonal immunoregulation effect (61). In general testosterone have an immunosuppressive effect and estrogen enhances the immunity. Females are less susceptible to viral infections (62).

Recent studies have shown that estrogen upregulates *ACE2* in human atrial myocardium by modulating the local Renin angiotensin aldosterone system (RAAS). Apart from *ACE2*, *Toll-like receptor (TLR) 7* is also encoded on X-chromosome. TLR7 mediates several immune cell responses (63). Berghöfer et al. showed that *in vitro* exposure of peripheral blood mononuclear cells (PBMCs) to TLR7 ligands results in higher production of interferon-α (IFNα) in cells from females compared to the cells from males (64).

The mechanisms by which androgens such as testosterone decrease the immune response has not been fully understood. Rettew et al. evaluated the acute effect of testosterone through *in vitro* treatment of macrophages generated in absence of androgen. The result was a significant decrease in *TLR4* expression and sensitivity to a TLR4-specific ligand. *In vivo* removal of testosterone resulted in significantly increased TLR4 cell surface expression and higher sensitivity to endotoxin. This may indicate an important mechanism of testosterone immunosuppressive effect (65).

Similar to the sex-based differences in SARAS-CoV2, some studies related to SARS-CoV infection have shown a higher mortality and severity of the disease in males. Karlberg et al. showed a significantly higher case fatality rate in males compared females infected with SARS-CoV ($p < 0.0001$) (52). Channappanavar et al. evaluated the susceptibility to SARS-CoV infection in male mice compared to the age-matched female group. Ovariectomy or estrogen receptor antagonist treatment of female mice showed increased mortality in the SARS-CoV infected mice indicating a protective effect of estrogen receptor signaling (66).

Although around 70% of health and social care workforce worldwide are women and they are in potential exposure to sick patients, most of the studies have shown a higher overall mortality among men with COVID-19. More research is needed to investigate how sex results in different outcomes during the COVID-19 pandemic (63).

This study has several limitations. Due to the rapidly emerging COVID-19 situation around the globe and the novelty of this coronavirus, there is still limited clinical data regarding diagnostic modalities and effective therapeutic measures. Most of the clinical findings were from observational studies. Future clinical trials and animal models are also required to have conclusive clinical information. More studies outside China are needed for comprehensive results that reflect COVID-19 epidemiology globally. Due to the lack of accurate reports of the new cases in different countries, the epidemiologic measures are also limited. As this pandemic is growing fast, future studies are needed for the evaluation of epidemiologic and clinical features of COVID-19.

CONCLUSION

COVID-19 has presented with a significant number of mortalities especially among the males around the world. The high rate of hospitalization and case fatality among hospitalized patients along with the lack of intensive care facilities necessitated the identification of the risk factors associated with severe disease and mortality. Males had a significant higher risk of mortality compared to females in our study which was higher than the previous reports from the studies done in China. The reason behind the gender and sex disparity in COVID-19 mortality is still unclear. COVID-19 has been an emerging, rapidly evolving situation. There is still a lot of unknown features of COVID-19 for the broad scientific community to study and identify the risk factors and possible causes of a worse outcome among these patients.

FUTURE DIRECTION

Further studies are essential on the role of sex hormones on mortality in COVID-19. Moreover, social, lifestyle, and environmental factors should be investigated to understand gender difference in COVID-19 mortality. Studying risk factors associated with mortality can assist us to develop a precise prognostic tool and to personalize treatment in COVID-19.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/supplementary material.

AUTHOR CONTRIBUTIONS

MN and MMi: conception and design of study. AT, YF, MA, SHas, PJ, and MN: acquisition of data. MN and MMi: analysis and/or interpretation of data. SHad, MMu, MN, and MMi: drafting and revision of manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

MN was supported by Department of Research of the School of Medicine (Grant number: 22960), Shahid Beheshti University of Medical Sciences, Tehran, Iran.

REFERENCES

1. Gates B. Responding to Covid-19 — a once-in-a-century pandemic? *N Engl J Med.* (2020) 382:1677–9. doi: 10.1056/NEJMp2003762

2. Coronavirus disease 2019. *(COVID-19) Situation Summary: Center for Disease Control and Prevention.* (2020). Available online at: https://www.cdc.gov/coronavirus/2019-ncov/summary.html (accessed March 12, 2020).

3. Peeri NC, Shrestha N, Rahman MS, Zaki R, Tan Z, Bibi S, et al. The SARS, MERS and novel coronavirus (COVID-19) epidemics, the newest and biggest global health threats: what lessons have we learned? *Int J Epidemiol.* (2020) dyaa033. doi: 10.1093/ije/dyaa033
4. Lukelsu. *Coronavirus Dashboard (GEOG 4046 Example).* (2020). Available online at: https://www.arcgis.com/apps/opsdashboard/index.html#/90b89509931e4c309e83d7f51e101a08 (accessed June 10, 2020).
5. Wu C, Chen X, Cai Y, Xia J, Zhou X, Xu S, et al. Risk factors associated with acute respiratory distress syndrome and death in patients with coronavirus disease 2019 pneumonia in Wuhan, China. *JAMA Intern Med.* (2020) 180:1–11. doi: 10.1001/jamainternmed.2020.0994
6. Alghamdi IG, Hussain, II, Almalki SS, Alghamdi MS, Alghamdi MM, El-Sheemy MA, et al. The pattern of Middle East respiratory syndrome coronavirus in Saudi Arabia: a descriptive epidemiological analysis of data from the Saudi Ministry of Health. *Int J Gen Med.* (2014) 7:417–23. doi: 10.2147/IJGM.S67061
7. Moher D, Liberati A, Tetzlaff J, Altman DG. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. *Ann Internal Med.* (2009) 151:264–9. doi: 10.7326/0003-4819-151-4-200908180-00135
8. Munn Z, Moola S, Lisy K, Riitano D. *The Joanna Briggs Institute Reviewers' Manual 2014. The Systematic Review of Prevalence and Incidence Data Adelaide.* Adelaide, SA: The Joanna Briggs Institute (2014).
9. Hui DS, Azhar E, Madani TA, Ntoumi F, Kock R, Dar O, et al. The continuing 2019-nCoV epidemic threat of novel coronaviruses to global health—the latest 2019 novel coronavirus outbreak in Wuhan, China. *Int J Infect Dis.* (2020) 91:264–6. doi: 10.1016/j.ijid.2020.01.009
10. Xia J, Tong J, Liu M, Shen Y, Guo D. Evaluation of coronavirus in tears and conjunctival secretions of patients with SARS-CoV-2 infection. *J Med Virol.* (2020) 92:589–94. doi: 10.1002/jmv.25725
11. Xu X-W, Wu X-X, Jiang X-G, Xu K-J, Ying L-J, Ma C-L, et al. Clinical findings in a group of patients infected with the 2019 novel coronavirus (SARS-Cov-2) outside of Wuhan, China: retrospective case series. *BMJ.* (2020) 368:m792. doi: 10.1136/bmj.m792
12. Zhang W, Du R-H, Li B, Zheng X-S, Yang X-L, Hu B, et al. Molecular and serological investigation of 2019-nCoV infected patients: implication of multiple shedding routes. *Emerg Microbes Infect.* (2020) 9:386–9. doi: 10.1080/22221751.2020.1729071
13. To KK-W, Tsang OTY, Chik-Yan Yip C, Chan K-H, Wu T-C, Chan J, et al. Consistent detection of 2019 novel coronavirus in saliva. *Clin Infect Dis.* (2020) ciaa149. doi: 10.1093/cid/ciaa149
14. Zou L, Ruan F, Huang M, Liang L, Huang H, Hong Z, et al. SARS-CoV-2 viral load in upper respiratory specimens of infected patients. *N Engl J Med.* (2020) 382:1177–9. doi: 10.1056/NEJMc2001737
15. Hoehl S, Berger A, Kortenbusch M, Cinatl J, Bojkova D, Rabenau H, et al. Evidence of SARS-CoV-2 infection in returning travelers from Wuhan, China. *N Engl J Med.* (2020) 382:1278–80. doi: 10.1056/NEJMc2001899
16. Pan Y, Zhang D, Yang P, Poon LL, Wang Q. Viral load of SARS-CoV-2 in clinical samples. *Lancet Infect Dis.* (2020) 20:411–2. doi: 10.1016/S1473-3099(20)30113-4
17. Tang N, Li D, Wang X, Sun Z. Abnormal coagulation parameters are associated with poor prognosis in patients with novel coronavirus pneumonia. *J Thrombosis Haemost.* (2020) 18:844–7. doi: 10.1111/jth.14768
18. Chung M, Bernheim A, Mei X, Zhang N, Huang M, Zeng X, et al. CT imaging features of 2019 novel coronavirus (2019-nCoV). *Radiology.* (2020) 295:202–7. doi: 10.1148/radiol.2020200230
19. Fang Y, Zhang H, Xie J, Lin M, Ying L, Pang P, et al. Sensitivity of chest CT for COVID-19: comparison to RT-PCR. *Radiology.* (2020) 200432. doi: 10.1148/radiol.2020200432
20. Guan W-j, Ni Z-y, Hu Y, Liang W-h, Ou C-q, He J-x, et al. Clinical characteristics of coronavirus disease 2019 in China. *N Engl J Med.* (2020) 382:1708–20. doi: 10.1101/2020.02.06.20020974
21. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet.* (2020) 395:497–506. doi: 10.1016/S0140-6736(20)30183-5
22. Kui L, Fang Y-Y, Deng Y, Liu W, Wang M-F, Ma J-P, et al. Clinical characteristics of novel coronavirus cases in tertiary hospitals in Hubei Province. *Chin Med J.* (2020) 133:1025–31. doi: 10.1097/CM9.0000000000000744
23. Li Q, Guan X, Wu P, Wang X, Zhou L, Tong Y, et al. Early transmission dynamics in Wuhan, China, of novel coronavirus–infected pneumonia. *N Engl J Med.* (2020) 382:1199–207. doi: 10.1056/NEJMoa2001316
24. Liu Y, Yang Y, Zhang C, Huang F, Wang F, Yuan J, et al. Clinical and biochemical indexes from 2019-nCoV infected patients linked to viral loads and lung injury. *Sci China Life Sci.* (2020) 63:364–74. doi: 10.1007/s11427-020-1643-8
25. Wang D, Hu B, Hu C, Zhu F, Liu X, Zhang J, et al. Clinical characteristics of 138 hospitalized patients with 2019 novel coronavirus–infected pneumonia in Wuhan, China. *JAMA.* (2020) 323:1061–9. doi: 10.1001/jama.2020.1585
26. Wu J, Liu J, Zhao X, Liu C, Wang W, Wang D, et al. Clinical characteristics of imported cases of COVID-19 in Jiangsu Province: a multicenter descriptive study. *Clin Infect Dis.* (2020) ciaa199. doi: 10.1093/cid/ciaa199
27. Zhang JJ, Dong X, Cao YY, Yuan YD, Yang YB, Yan YQ, et al. Clinical characteristics of 140 patients infected by SARS-CoV-2 in Wuhan, China. *Allergy.* (2020) 75:1730–41. doi: 10.1111/all.14238.
28. Ai T, Yang Z, Hou H, Zhan C, Chen C, Lv W, et al. Correlation of chest CT and RT-PCR testing in coronavirus disease 2019. (COVID-19) in China: a report of 1014 cases. *Radiology.* (2020) 200642. doi: 10.1148/radiol.2020200642
29. Pan F, Ye T, Sun P, Gui S, Liang B, Li L, et al. Time course of lung changes on chest CT during recovery from 2019 novel coronavirus (COVID-19) pneumonia. *Radiology.* (2020) 295:715–21. doi: 10.1148/radiol.2020200370
30. Zhu N, Zhang D, Wang W, Li X, Yang B, Song J, et al. A novel coronavirus from patients with pneumonia in China, 2019. *N Engl J Med.* (2020) 382:727–33. doi: 10.1056/NEJMoa2001017
31. Yang X, Yu Y, Xu J, Shu H, Liu H, Wu Y, et al. Clinical course and outcomes of critically ill patients with SARS-CoV-2 pneumonia in Wuhan, China: a single-centered, retrospective, observational study. *Lancet Respir Med.* (2020) 8:475–81. doi: 10.1016/S2213-2600(20)30079-5
32. Bajema KL, Oster AM, McGovern OL, Lindstrom S, Stenger MR, Anderson TC, et al. Persons evaluated for 2019. Novel coronavirus—United States, january 2020. *Morb Mortal Week Rep.* (2020) 69:166–70. doi: 10.15585/mmwr.mm6906e1
33. Bernheim A, Mei X, Huang M, Yang Y, Fayad ZA, Zhang N, et al. Chest CT findings in coronavirus disease-19 (COVID-19): relationship to duration of infection. *Radiology.* (2020) 295:3. doi: 10.1148/radiol.2020200463
34. Chen N, Zhou M, Dong X, Qu J, Gong F, Han Y, et al. Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study. *Lancet.* (2020) 395:507–13. doi: 10.1016/S0140-6736(20)30211-7
35. Pan Y, Guan H, Zhou S, Wang Y, Li Q, Zhu T, et al. Initial CT findings and temporal changes in patients with the novel coronavirus pneumonia (2019-nCoV): a study of 63 patients in Wuhan, China. *Eur Radiol.* (2020) 30:3306–9. doi: 10.1007/s00330-020-06731-x
36. Xu Y-H, Dong J-H, An W-m, Lv X-Y, Yin X-P, Zhang J-Z, et al. Clinical and computed tomographic imaging features of novel coronavirus pneumonia caused by SARS-CoV-2. *J Infection.* (2020) 80:394–400. doi: 10.1016/j.jinf.2020.02.017
37. Xu X, Yu C, Qu J, Zhang L, Jiang S, Huang D, et al. Imaging and clinical features of patients with 2019 novel coronavirus SARS-CoV-2. *Eur J Nuclear Med Mol Imag.* (2020) 47:1275–80. doi: 10.1007/s00259-020-04735-9
38. Chang D, Lin M, Wei L, Xie L, Zhu G, Cruz CSD, et al. Epidemiologic and clinical characteristics of novel coronavirus infections involving 13 patients outside Wuhan, China. *JAMA.* (2020) 323:1092–3. doi: 10.1001/jama.2020.1623
39. Chen W, Lan Y, Yuan X, Deng X, Li Y, Cai X, et al. Detectable 2019-nCoV viral RNA in blood is a strong indicator for the further clinical severity. *Emerg Microbes Infect.* (2020) 9:469–73. doi: 10.1080/22221751.2020.1732837
40. Kwok KO, Wong V, Wei VWI, Wong SYS, Tang JW-T. Novel coronavirus (2019-nCoV) cases in Hong Kong and implications for further spread. *J Infect.* (2020) 80:671–93. doi: 10.1016/j.jinf.2020.02.002
41. Ihle-Hansen H, Berge T, Tveita A, Rønning EJ, Ernø PE, Andersen EL, et al. COVID-19: symptoms, course of illness and use of clinical scoring systems for the first 42 patients admitted to a Norwegian local hospital. *Tidsskr Nor Legeforen.* (2020) 140:7. doi: 10.4045/tidsskr.20.0301
42. Yu Y, Xu D, Fu S, Zhang J, Yang X, Xu L, et al. Patients with COVID-19 in 19 ICUs in Wuhan, China: a cross-sectional study. *Crit Care.* (2020) 24:219. doi: 10.1186/s13054-020-02939-x

43. Zhou F, Yu T, Du R, Fan G, Liu Y, Liu Z, et al. Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: a retrospective cohort study. *Lancet.* (2020) 395:1054–62. doi: 10.1016/S0140-6736(20)30566-3
44. Greaves K, Oxford JS, Price CP, Clarke GH, Crake T. The prevalence of myocarditis and skeletal muscle injury during acute viral infection in adults: measurement of cardiac troponins I and T in 152 patients with acute influenza infection. *Arch Internal Med.* (2003) 163:165–8. doi: 10.1001/archinte.163.2.165
45. Gao C, Wang Y, Gu X, Shen X, Zhou D, Zhou S, et al. Association between cardiac injury and mortality in hospitalized patients infected with avian influenza A (H7N9) virus. *Crit Care Med Soc.* (2020) 48:451–8. doi: 10.1097/CCM.0000000000004207
46. Ludwig A, Lucero-Obusan C, Schirmer P, Winston C, Holodniy M. Acute cardiac injury events </=30 days after laboratory-confirmed influenza virus infection among U.S. veterans, 2010-2012. *BMC Cardiovasc Disord.* (2015) 15:109. doi: 10.1186/s12872-015-0095-0
47. Feng D, Jia N, Fang L-Q, Richardus JH, Han X-N, Cao W-C, et al. Duration of symptom onset to hospital admission and admission to discharge or death in SARS in mainland China: a descriptive study. *Trop Med Int Health.* (2009) 14:28–35. doi: 10.1111/j.1365-3156.2008.02188.x
48. Qian K, Deng Y, Tai Y, Peng J, Peng H, Jiang L. Clinical characteristics of 2019. novel infected coronavirus pneumonia:a systemic review and meta-analysis. *medRxiv [Preprint]*. (2020). doi: 10.1101/2020.02.14.20021535
49. Li L-Q, Huang T, Wang Y-Q, Wang Z-P, Liang Y, Huang T-B, et al. 2019 novel coronavirus patients' clinical characteristics, discharge rate and fatality rate of meta-analysis. *J Med Virol.* (2020) 92:577–83. doi: 10.1002/jmv.25757
50. Rodriguez-Morales AJ, Cardona-Ospina JA, Gutiérrez-Ocampo E, Villamizar-Peña R, Holguin-Rivera Y, Escalera-Antezana JP, et al. Clinical, laboratory and imaging features of COVID-19: a ystematic review and meta-analysis. *Travel Med Infect Dis.* (2020) 34:101623. doi: 10.1016/j.tmaid.2020.101623
51. Sun Q, Qiu H, Huang M, Yang Y. Lower mortality of COVID-19 by early recognition and intervention: experience from Jiangsu Province. *Ann Intensive Care.* (2020) 10:33. doi: 10.1186/s13613-020-00650-2
52. Karlberg J, Chong DS, Lai WY. Do men have a higher case fatality rate of severe acute respiratory syndrome than women do? *Am J Epidemiol.* (2004) 159:229–31. doi: 10.1093/aje/kwh056
53. Klein SL. The effects of hormones on sex differences in infection: from genes to behavior. *Neurosci Biobehav Rev.* (2000) 24:627–38. doi: 10.1016/S0149-7634(00)00027-0
54. Yang T, Barnett R, Jiang S, Yu L, Xian H, Ying J, et al. Gender balance and its impact on male and female smoking rates in Chinese cities. *Soc Sci Med.* (2016) 154:9–17. doi: 10.1016/j.socscimed.2016.02.035
55. Leung JM, Yang CX, Tam A, Shaipanich T, Hackett TL, Singhera GK, et al. ACE-2 Expression in the small airway epithelia of smokers and COPD patients: implications for COVID-19. *medRxiv [Preprint]*. (2020). doi: 10.1101/2020.03.18.20038455
56. Ritchie H. *Who Smokes More, Men or Women?* (2019). Available online at: https://ourworldindata.org/who-smokes-more-men-or-women (accessed September 02, 2019).
57. Li X, Xu S, Yu M, Wang K, Tao Y, Zhou Y, et al. Risk factors for severity and mortality in adult COVID-19 inpatients in Wuhan. *J Allergy Clin Immunol.* (2020) 146:110–8. doi: 10.1016/j.jaci.2020.04.006
58. Ruan Q, Yang K, Wang W, Jiang L, Song J. Clinical predictors of mortality due to COVID-19 based on an analysis of data of 150 patients from Wuhan, China. *Intensive Care Med.* (2020) 46:846–8. doi: 10.1007/s00134-020-05991-x
59. Simonnet A, Chetboun M, Poissy J, Raverdy V, Noulette J, Duhamel A, et al. High prevalence of obesity in severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) requiring invasive mechanical ventilation. *Obesity.* (2020) 28:1195–9. doi: 10.1002/oby.22831
60. Engin AB, Engin ED, Engin A. Two important controversial risk factors in SARS-CoV-2 infection: obesity and smoking. *Environ Toxicol Pharmacol.* (2020) 78:103411. doi: 10.1016/j.etap.2020.103411
61. Tay MZ, Poh CM, Rénia L, MacAry PA, Ng LFP. The trinity of COVID-19: immunity, inflammation and intervention. *Nat Rev Immunol.* (2020) 20:363–74. doi: 10.1038/s41577-020-0311-8
62. Taneja V. Sex hormones determine immune response. *Front Immunol.* (2018) 9:1931. doi: 10.3389/fimmu.2018.01931
63. Gebhard C, Regitz-Zagrosek V, Neuhauser HK, Morgan R, Klein SL. Impact of sex and gender on COVID-19 outcomes in Europe. *Biol Sex Differ.* (2020) 11:29. doi: 10.1186/s13293-020-00304-9
64. Berghöfer B, Frommer T, Haley G, Fink L, Bein G, Hackstein H. TLR7 ligands induce higher IFN-alpha production in females. *J Immunol.* (2006) 177:2088–96. doi: 10.4049/jimmunol.177.4.2088
65. Rettew JA, Huet-Hudson YM, Marriott I. Testosterone reduces macrophage expression in the mouse of toll-like receptor 4, a trigger for inflammation and innate immunity. *Biol Reprod.* (2008) 78:432–7. doi: 10.1095/biolreprod.107.063545
66. Channappanavar R, Fett C, Mack M, Ten Eyck PP, Meyerholz DK, Perlman S. Sex-based differences in susceptibility to severe acute respiratory syndrome coronavirus infection. *J Immunol.* (2017) 198:4046–53. doi: 10.4049/jimmunol.1601896

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 21 July 2020
doi: 10.3389/fimmu.2020.01841

Mechanisms Underlying Potential Therapeutic Approaches for COVID-19

Abdelouaheb Benani[1] *and Sanae Ben Mkaddem*[2,3]*

[1] Unité de Biologie Moléculaire, Institut Pasteur du Maroc, Casablanca, Morocco, [2] U978 Institut National de la Santé et de la Recherche Médicale, Bobigny, France, [3] UFR SMBH, Université Sorbonne Paris Nord, Bobigny, France

Coronavirus disease (COVID-19) is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which is a betacoronavirus, and is associated with cytokine storm inflammation and lung injury, leading to respiratory distress. The transmission of the virus is mediated by human contact. To control and prevent the spread of this virus, the majority of people worldwide are facing quarantine; patients are being subjected to non-specific treatments under isolation. To prevent and stop the COVID-19 pandemic, several clinical trials are in the pipeline. The current clinical trials either target the intracellular replication and spread of the virus or the cytokine storm inflammation seen in COVID-19 cases during the later stages of the disease. Since both targeting strategies are different, the window drug administration plays a crucial role in the efficacy of the treatment. Here, we review the mechanism underlying SARS-CoV-2 cell infection and potential future therapeutic approaches.

Keywords: SARS-CoV-2, COVID-19, immune therapy, monoclonal antibody, respiratory distress, cytokine treatment

OPEN ACCESS

Edited by:
Zisis Kozlakidis, International Agency for Research on Cancer (IARC), France

Reviewed by:
Talia H. Swartz, Icahn School of Medicine at Mount Sinai, United States
Vijayakumar Velu, Emory University, United States

***Correspondence:**
Sanae Ben Mkaddem
sanae.benmkaddem@inserm.fr

Specialty section:
This article was submitted to Viral Immunology, a section of the journal Frontiers in Immunology

Received: *24 April 2020*
Accepted: *08 July 2020*
Published: *21 July 2020*

Citation:
Benani A and Ben Mkaddem S (2020) Mechanisms Underlying Potential Therapeutic Approaches for COVID-19. Front. Immunol. 11:1841. doi: 10.3389/fimmu.2020.01841

INTRODUCTION

The members of the Coronaviridae family cause mild respiratory disease, and infection with these viruses can be transmitted between humans (1). Severe acute respiratory syndrome coronavirus (SARS-CoV) is transmitted from animals to humans, leading to severe respiratory diseases in individuals (2). SARS was discovered in Guangdong Province, China, in 2002 (3). Chinese bats serve as the natural reservoir hosts of SARS-CoV-2 (4). The human transmission of SARS-CoV requires intermediate hosts, such as animal food sources, including pangolin and cats (5). No specific antivirals or effective vaccines are available to treat or prevent SARS. In 2002 and 2003, the SARS pandemic was controlled by travel restrictions and patient isolation.

Recently, a new virus strain from the same virus family was discovered in Wuhan, Hubei Province, China, that causes coronavirus disease 19 (COVID-19) (6). It has been suggested that the human transmission of this strain was linked to the Hunan seafood market. The infection is very contagious and results in the development of the disease and fatalities (7). SARS-CoV-2 is closely related to SARS-CoV, and COVID-19 has been described as a new lung disease (8). Infections have also been detected in several countries globally and are linked to international travel. Elucidating the mechanisms through which the virus gains entry into target cells and how this process can be inhibited would allow the development of new therapeutics or vaccines to rapidly curb the ongoing pandemic. A significant number of clinical trials have been started to explore potential therapeutic strategies for COVID-19 to identify as quickly as possible high-quality

efficient treatments to stop the ongoing pandemic. Here, we present a brief overview of the SARS-CoV infection mechanism and potential strategies to prevent virus entry along with the effects of infection, such as inflammatory cytokine storms, on lung injury. We discuss some published data and the mechanism of the ongoing clinical trials.

MECHANISM OF SARS-COV CELL INFECTION

Basically, the entry of coronavirus is mediated by the interaction of cellular receptor proteins and the S1 unit of the viral spike (S) protein, which, in turn, promotes viral attachment to the target cell surface. Furthermore, viral attachment requires cellular proteases to prime the S protein, which entails its cleavage at the S1/S2 and S2' sites, resulting in the fusion of the viral and cellular membranes. It has been shown that the S protein from SARS interacts with angiotensin-converting enzyme 2 (ACE2) as its receptor and uses the cellular serine protease TMPRSS2 to prime the S protein (9, 10). Additionally, it has been demonstrated that the SARS-S/ACE2 interaction favors the spread of the virus, leading to severe acute respiratory syndrome (11). ACE and ACE2 have high homology with metalloproteases that play a role in the renin-angiotensin system (RAS) to maintain blood pressure homeostasis. The renin protease cleaves angiotensinogen to generate angiotensin I (Ang I). The two C-terminal amino acids of Ang I are cleaved by ACE to generate angiotensin II (Ang II), whereas ACE2 cleaves Ang II. Ang II acts specifically through Ang II receptor type 1 (AT1R) and Ang II receptor type 2 (AT2R) (12, 13). ACE also degrades additional substrates such as bradykinin or apelin (14). ACE2 has been identified as the key determinant of SARS-CoV transmissibility (15). The SARS-S and SARS-2-S proteins have 76% amino acid homology. However, it is not yet clear whether SARS-2-S and SARS-S use ACE2 and TMPRSS2 for host cell binding. A recent study demonstrated that SARS-CoV-2 uses the same ACE2 receptor as SARS-CoV to enter the target cell and also uses the same cellular protease, the serine protease TMPRSS2, to prime the S protein. The study also suggested a treatment strategy based on the inhibition of S protein priming by targeting TMPRSS2 to block entry. Moreover, the study showed that sera from convalescent SARS patients cross-neutralized the S protein to block SARS-2 entry (3).

COVID-19 AND CYTOKINE STORM SYNDROME

The antiviral response is mediated by both innate and acquired immunity, which recognize pathogen-associated molecular patterns (PAMPs) and the antigen-specific adaptive immune response. The viral response is based on the release of inflammatory mediators (cytokines, chemokines, leukotrienes, proteases, and reactive oxygen species) and on the clearance of virus through internalization and killing of the virus. Cell responses are in many ways controlled by the balance between antagonistic signals, which may affect the immune response to pathogens. The resulting balance is of great importance to prevent damage to tissues through immunopathology and to ensure the return of activated cells to a resting state. However, exaggerated and excessive synthesis of cytokines can lead to an acute, severe systemic inflammatory response known as a "cytokine storm" and cause severe damage to multiple organs (16). The cytokine profile of COVID-19 patients with differences in disease severity has been investigated, and a subset of patients with severe COVID-19 develop profound inflammation and multiorgan dysfunction that is consistent with a "cytokine storm." Recently, a large panel of cytokines (IFN-γ, TNF-α, IL-2, IL-4, IL-6, and IL-10) and C-reactive protein (CRP) have been analyzed and compared with serum samples from a control group and from COVID-19 patients. The values for cytokines and CRP were significantly higher in patients with COVID-19 than those in healthy controls. However, using univariate logistic regression analysis, only two cytokines, IL-6 and IL-10, were found to be predictive of disease severity, suggesting that a higher level of cytokine storm is associated with severe disease development. Improving the understanding of hypercytokinemia (i.e., IL-6 levels from 100 to 5,000 pg/mL) and immune dysregulation associated with COVID-19 is urgent. Investigations of different potential therapeutic strategies for COVID-19 cytokine storm syndrome are ongoing that use corticosteroids, IL-6 blockade and IL-1 inhibition (17). A series of clinical trials of IL-6 inhibitors such as tocilizumab, sarilumab and siltuximab are also underway (see **Table 1**).

COAGULATION AND FIBRINOLYSIS IN COVID-19

The host defense against viral infection activates the coagulation cascade to limit the spread of pathogens. During the first phase of infection, an adaptive haemostatic response occurs that is associated with the activation of a systemic inflammatory response, which is characterized by an increase in inflammatory activity and thrombin and fibrinogen generation. The increase in cytokine production during virus infection induces additional procoagulant effects, such as the expression of tissue factors that are major initiators of coagulation activation. Moreover, other factors, such as DAMPs and neutrophil extracellular traps, may also contribute to the procoagulant profile in COVID-19. During pulmonary infection, the measurement of coagulation and fibrinolysis factors in bronchoalveolar lavage fluid has demonstrated an increase in thrombin generation, an insufficient balance in physiologic anticoagulation, and the suppression of fibrinolysis, mediating the pathogenesis of respiratory distress. Endothelial injury of the pulmonary capillary is also caused by vascular endothelial damage. SARS-CoV-2 infects endothelial cells through the ACE2 receptor, and viral spread and rapid viral replication leads to massive endothelial cell apoptosis and inhibits the anticoagulant function of the vascular lumen. Moreover, endothelial dysfunction contributes to procoagulant changes in COVID-19 (42).

Platelets play a dual role; they contribute to haemostasis but also to inflammation and the host defense response, especially

TABLE 1 | Current therapeutic drugs used to treat COVID-19.

Drug	Description and mechanism of action	References
Inhibitors of the cellular entry of SARS-CoV-2		
Chloroquine and Hydroxychloroquine (Quensyl™, Plaquenil™, Hydroquin™, Dolquine™, Quinoric™)	• Antimalarial; they have been used for decades for the prophylaxis and treatment of malaria and for various autoimmune diseases • Inhibit the terminal phosphorylation of ACE2 and elevate the pH in endosomes. • Chloroquine can inhibit the entry of SARS-CoV-2 and prevent virus-cell fusion by interfering with glycosylation of the ACE2 receptor and its binding with the spike protein, suggesting that chloroquine treatment might be more effective in the early stage of infection before COVID-19 reduces ACE2 expression and activity. • Hydroxychloroquine exhibits an anti-inflammatory effect on Th17-related cytokines (IL-6, IL-17, and IL-22) in healthy individuals and systemic lupus erythematosus and rheumatoid arthritis patients.	(18–21)
Camostat mesylate (Foipan™)	• Developed decades ago for the treatment of oral squamous cell carcinoma, dystrophic epidermolysis, exocrine pancreatic enzyme inhibition, and chronic pancreatitis • TMPRSS2 protease activity as a synthetic serine protease inhibitor. In a clinical trial investigating the effects of camostat mesylate against dyspepsia associated with non-alcoholic mild pancreatic disease, 95 patients received 200 mg camostat mesylate three times daily for 2 weeks and showed only mild side effects and no severe adverse effects.	(22–24)
Nafamostat mesylate (Buipel™)	• Approved in Japan for the treatment of acute pancreatitis, disseminated intravascular coagulation and for anticoagulation in extracorporeal circulation • TMPRSS2 protease activity: clinically proven as a synthetic serine protease inhibitor. Nafamostat mesylate has been shown to inhibit MERS-CoV S protein-mediated viral membrane fusion with TMPRSS2-expressing lung Calu-3 host cells by inhibiting TMPRSS2 protease activity. It may also inhibit the cellular entry of SARS-CoV-2. In cell culture experiments with simian Vero E6 cells infected with SARS-CoV-2, Nafamostat mesylate was shown to inhibit SARS-CoV-2 infection at an EC_{50} of 22.50 μM.	(19, 25, 26)
Monoclonal antibodies targeting SARS-CoV entry		
80R, F26G19, m396, CR3014, CR3022, F26G18, m396, 201, S230	• Binds to the conformational epitope on the S1 fragment of SARS-CoV or to the amino acid residues with high affinity on the S1 fragment of SARS-CoV. • Blocks the interaction of the S1 subunit protein with the cellular receptor ACE2	(27–32)
Inhibitors of the replication, membrane fusion, and assembly of SARS-CoV-2		
Remdesivir	• A novel small-molecule adenine nucleotide analog antiviral drug synthesized and developed by Gilead Sciences in 2017 that has shown efficacy against Ebola virus in rhesus monkeys. It displays antiviral activity against other single-stranded RNA viruses, including filoviruses, pneumoviruses, paramyxoviruses, and the coronaviruses MERS-CoV and SARS-CoV. • It results in the delayed chain cessation of nascent viral RNA. It potently blocks SARS-CoV-2 infection at a low range of micromolar concentrations and has a high selectivity index with an EC50 of 0.77 μM and a CC50 > 100 μM. It acts early in infection and is metabolized into its active form GS-441524, which is an adenine nucleotide analog that interferes with the activity of viral RNA polymerase and that promotes the evasion of proofreading by viral exoribonuclease, leading to the inhibition of viral RNA synthesis.	(19, 33, 34)
Lopinavir/ritonavir (Kaletra™)	• Lopinavir was developed in 1998 to circumvent HIV resistance toward the protease inhibitor ritonavir. The combination of lopinavir and ritonavir was first established as an effective oral drug for the treatment of HIV-infected individuals when used in combination with other antiretroviral agents. • Lopinavir-ritonavir administration significantly decreased coronavirus titres, and low or no coronavirus titres were observed in the follow-up study. Another study investigated lopinavir in patients with COVID-19 receiving either lopinavir-ritonavir 400 mg/100 mg orally twice daily plus the standard of care or the standard of care alone.	(35)
Umifenovir (Arbidol™)	• A small indole-derivate molecule licensed for oral prophylaxis and treatment of infections with influenza A and B viruses and other respiratory viruses that has been demonstrated to inhibit *in vitro* infection with globally prevalent pathogenic viruses, including the hepatitis C virus, hepatitis B virus, Ebola virus, Lassa virus, human herpesvirus, poliovirus, and vesicular stomatitis virus. • Prevents viral host cell entry by inhibiting membrane fusion of the viral envelope and the host cell cytoplasmic membrane via inhibition of clathrin-mediated endocytosis, thereby preventing virus infection.	(36)
Favipiravir (Avigan™)	• An oral pyrazinecarboxamide derivative and guanine analog. • Selectively and potently inhibits the RNA-dependent RNA polymerase (RdRP) of RNA viruses (influenza A virus, flavi-, alpha-, filo-, bunya-, arena-, and noroviruses as well as West Nile virus, yellow fever virus, foot-and-mouth-disease virus, Ebola virus and Lassa virus) and induces lethal RNA transversion mutations, thereby producing a nonviable virus phenotype. A study showed favipiravir has efficacy in Vero E6 cells infected with SARS-CoV-2 with an EC50 of 61.88 μM and a CC50 over 400 μM.	(19)
Anti-cytokines and chemokines		
Tocilizumab Sarilumab Siltuximab	• Anti-IL-6 receptor is a human immunoglobulin G1 monoclonal antibody (mAb) that binds specifically to both soluble and membrane-bound interleukin-6 receptors (IL-6Rs) • Blocks the interaction between the cytokine and its receptor, avoiding the amplification of inflammation associated with lung injury that leads to respiratory distress.	(37, 38).

(Continued)

TABLE 1 | Continued

Drug	Description and mechanism of action	References
Supporting agents		
Azithromycin	• An antibiotic that can be used for different types of bacterial infections, such as respiratory and skin infections and sexually transmitted diseases. It has been proven to be active against the Zika and Ebola viruses and to prevent severe respiratory tract infections when used to treat patients suffering from viral infection. It has been used as an adjunctive therapy to provide antibacterial coverage and exerts potential immunomodulatory and anti-inflammatory effects in the treatment of some viral respiratory tract infections (e.g., influenza). • Prevents the growth of bacteria by interfering with bacterial protein synthesis. It binds to the 50S subunit of the bacterial ribosome, thus inhibiting the translation of mRNA. In COVID-19 patients, Gautret et al. reported 100% viral clearance based on nasopharyngeal swabs in six patients who were co-treated with hydroxychloroquine and azithromycin. However, Molina et al. reported findings that contrasted with those reported by Gautret. Based on those results, the data presented to date are insufficient to evaluate the possible clinical benefits of azithromycin in patients with COVID-19	(39, 40)
Corticosteroids	• A potent anti-inflammatory and anti-fibrotic drug. • Low doses of methylprednisolone prevent extended cytokine release and may accelerate the resolution of pulmonary and systemic inflammation in pneumonia. Recently, many medical researchers have stated that corticosteroids may improve the dysregulated immune response caused by sepsis (a possible complication of infection with COVID-19) and increase the blood pressure when it is low. In a retrospective cohort study, 201 patients with confirmed COVID-19 who developed ARDS were treated with methylprednisolone (1–2 mg/kg daily via IV for 5–7 days), and the results showed that treatment with methylprednisolone may be beneficial for patients who develop ARDS in terms of the reduction of the risk of death.	(41)

during lung infection. Recently, many cases of thrombocytopenia have been observed in COVID-19 patients, and the baseline platelet levels and changes were associated with subsequent mortality. However, the mechanism of SARS-CoV-2 involved in thrombocytopenia is not yet clear (43). One of the possibilities is that lung tissue injury could cause platelet activation and aggregation, and thrombi formation at the site of the injury may lead to the consumption of platelets and megakaryocytes. In addition, SARS-CoV-2 induces increases in D-dimer and fibrinogen and further increases the consumption of platelets in damaged lungs.

POTENTIAL THERAPEUTIC APPROACHES

Monoclonal Antibodies Targeting SARS-CoV Entry

The spike proteins of SARS-CoV-2 play a major role in the interaction between the virus and the ACE2 receptor expressed by the host cell. The binding of the spike protein to ACE2 leads to membrane fusion and the initiation of the viral life cycle. To inhibit SARS-CoV-2 binding to ACE2, several neutralizing monoclonal antibodies (MAbs) targeting the spike protein of SARS-CoV-2 have been developed. Among them, the 80R MAb binds to the S1 fragment of SARS-CoV at the conformational epitope (amino acid residues 426–492) and blocks the binding of the viral S1 subunit to the ACE2 receptor, thereby preventing the entry and spread of the virus (44). These findings have been demonstrated by *in vitro* and *in vivo* studies (27, 45). Other MAbs targeting different epitopes of the S1 subunit have also been developed and tested by *in vitro* and *in vivo* studies, such as CR3022, F26G18, F26G19, m396, 1A9, and CR3014 (27–32).

A recent study suggested the involvement of similar mechanisms of host entry in infection with SARS-CoV-2, and consequently, different studies are currently investigating single MAbs or combinations of different MAbs. Such antibodies recognize different epitopes on the SARS-CoV-2 surface, which should be assessed first by *in vitro* and *in vivo* (mouse) approaches prior to different clinical trials. However, several neutralizing MAbs also bind to IgG Fc receptors (FcγR). The antibody/FcγR interaction might lead to virus entry that could infect other cells expressing this receptor independently of the ACE2-specific virus receptor. Recently, it has been demonstrated that FcγRIIA plays a major role in viral entry via antibody-dependent enhancement (ADE) using *in vitro* strategies (46). However, the signaling pathway associated with the MAbs/virus/receptor interaction is not yet clear. ADE viral entry in the presence of neutralizing MAbs has been demonstrated for many viruses, especially for those expressing the coronavirus spike protein. Understanding the effect of this interaction on the activation of human cells expressing the Fc receptor and viral proliferation may help to establish new vaccination strategies in the future.

Treatment of Inflammatory Cytokine Storm

MAbs Against the IL-6 Receptor

To explore the pathophysiological mechanisms and development of novel therapeutic approaches for sepsis, a recent study using caecal ligation and puncture (CLP) was performed in a septic mouse model. The mouse models demonstrated classical inflammatory symptoms associated with an increase in soluble triggering receptors expressed on immune cells, including interleukin (IL)-6, IL-10, TNF-α, macrophage inflammatory protein (MIP)-1α, MIP-1β, and MIP-2. These results were similar to those found in human patients with sepsis (47). IL-6 plays an important role in host defense during infections. However, exacerbation of IL-6 production favors acute severe systemic inflammation, which is named 'cytokine storm' (48). During the COVID-19 pandemic, a recent study explored the levels of cytokines, including IL-6, and the T cell frequency in three

groups of individuals: healthy individuals and patients with moderate and severe COVID-19 cases. The moderate cases presented an increase in IL-6 and a decrease in the total T lymphocyte frequency. However, the severe COVID-19 cases showed an increase in IL-6, IL-2R, IL-10, and TNFα secretion associated with a severe decrease in T cells, particularly CD4+ T cells (49). These results suggest that IL-6 plays a key role in the amplification of inflammation associated with lung injury, leading to respiratory distress (37, 38). Moreover, this antibody has been used in the treatment of rheumatoid arthritis and was approved by the FDA 10 years ago, and the side effects have been extensively studied (50). Taken together, these findings suggest that IL-6 or its receptor present a potent target of interest for the treatment of COVID-19-associated acute respiratory distress syndrome (ARDS). In this context, treatment of one case of COVID-19 associated with respiratory failure with an anti-interleukin-6 receptor inhibitor named tocilizumab resulted in favorable recovery (51). To explore whether tocilizumab can be used as a treatment for COVID-19, clinical trials with a large number of patients with the correct groups should be conducted robustly to prevent mortality. However, the optimal disease stage for the administration of tocilizumab must be defined carefully. Since it has been shown that IL-6 can either suppress or facilitate viral replication (52), one crucial issue to address will be the optimal timing of anti-IL6 administration. If it occurs too early, the drugs may affect viral clearance. If it occurs too late, the drugs may not be effective. The optimal timing of the administration of anti-IL-6 must be assessed in trials. Several randomized controlled trials of tocilizumab, sarilumab and siltuximab, either alone or in combination, are now being proposed in patients with severe COVID-19 and are underway mainly in China, Western Europe, USA, Russia, Malaysia, and Australia (53). Moreover, different clinical trials are under way to evaluate the safety and efficacy of IL-6 inhibitors with various protocols and comparators. The identifiers of the clinical trials are NCT04332913, NCT04335071, NCT04317092, NCT04324073, NCT04320615, NCT04306705, NCT04315298, NCT04315480, NCT04321993, NCT04348500, NCT04329650, NCT04330638, NCT04345289, NCT04327388, NCT04341870, and NCT04322773 (ClinicalTrials.gov).

MAbs Against Chemokine Receptors

Several clinical trials are also ongoing to examine the effect of blocking other proinflammatory cytokines, such as TNF (54) and granulocyte–macrophage colony-stimulating factor (GM–CSF), with the clinical trial identifier NCT04341116. The aim of this study is to interfere with cytokine signaling, leading to decreased hyperinflammation in patients with severe COVID-19. Indeed, the most highly pathological macrophages are derived from the circulating monocytes infiltrating the lung. Moreover, CCR2 plays a central role in the recruitment and accumulation of monocytes in inflamed tissues (55). Altogether, these results suggest that CCR2 blockade could potentially help to reduce the accumulation of pathological monocytes in inflamed tissues. A new clinical trial (NCT04343651) targeting CCR5, another chemokine receptor that regulates monocyte and T cell recruitment, is ongoing in patients with COVID-19 with mild-to-moderate symptoms of respiratory illness.

Chloroquine

Chloroquine (CQ) or hydroxychloroquine (HCQ) (a more soluble and less toxic metabolite of CQ) are antimalarial products that have been tested in humans (56). CQ and HCQ are also used in the treatment of several autoimmune diseases, such as systemic lupus erythematosus and rheumatoid arthritis. Additionally, CQ inhibits autophagy, favoring the apoptosis of cancer cells (57).

Promising studies have demonstrated that the CQ/HCQ compounds have the ability to inhibit certain coronaviruses, such as SARS-CoV-1 (58). Additional *in vitro* studies have shown that CQ and HCQ have antiviral activity against SARS-CoV-2, with more side effects being observed for CQ than for HCQ (58). In contrast, others have demonstrated that HCQ has greater antiviral activity than CQ during SARS-CoV-2 infection (18). Basically, CQ or HCQ exert their effects on eukaryotic cells by increasing the vacuolar pH of organelles such as endosomes and lysosomes. The increase in pH neutralizes the acidic lysosomal pH, decreasing autophagosome-lysosome fusion and autophagic degradation (59, 60). Autophagosome-lysosome fusion is essential for virus/cell fusion and immune-modulating activity (61). CQ and HCQ can also modify the glycosylation of ACE2, which binds to the spike protein S of SARS-CoV. This may interfere with the virus-receptor interaction (19). Additionally, an *in vitro* approach demonstrated that CQ inhibits COVID-19 virus infection (62). Some studies have indicated that HCQ also reduces the levels of some proinflammatory cytokines, such as IL-6, IL-18, and TNF-α (63). Indeed, CQ and HCQ inhibit endosomal TLRs and have anti-inflammatory effects by inhibiting prostaglandin synthesis or lipid peroxidation (64).

Hence, it was suggested that CQ and HCQ represent a potential new drug treatment for COVID-19. However, there are some limitations in performing clinical trials in patients owing to the restrictions on research studies using cell culture or animals and side effects, such as cardiotoxicity and liver cytotoxicity, due to the half-life of these compounds of ∼3.1 days (65). However, the risk of toxicity in patients treated for 10 years with HCQ for systemic lupus erythematosus was shown to be approximately 7.5% and to be higher in patients treated for longer periods (66). In COVID-19-associated acute infection, CQ and HCQ are used for a very short time (∼5 days). Nevertheless, acute adverse events, such as hypersensitivity and gastrointestinal intolerance, require attention, especially in critically ill patients who may develop similar clinical manifestations due to COVID-19. Additionally, CQ and HCQ can be safely used during pregnancy (67). Recently, a clinical trial with a small sample size showed that HCQ treatment is associated with a decrease in viral load in COVID-19 patients, and the effect is reinforced by azithromycin (39). Because of the low number of patients and the lack of some group controls during this recent study, new national and international clinical trials are being conducted to confirm the authenticity of these findings. A current clinical trial of CQ and HCQ therapy in the treatment of COVID-19 in Europe may reveal new possibilities for antiviral therapy for

COVID-19 to stop the pandemic. Although the antiviral activity of hydroxychloroquine remains uncertain, there have been several controversies regarding the clinical benefits of this drug in patients with COVID-19. Recently, a new publication showed the beneficial effects of hydroxychloroquine or chloroquine when used alone or with a macrolide on in-hospital outcomes for COVID-19. Each of the drug regimens was associated with a decrease in in-hospital survival and an increased frequency of ventricular arrhythmias when used for the treatment of COVID-19. However, this study was retracted from the Lancet journal (https://doi.org/10.1016/S0140-6736(20)31174-0). In contrast, an approved study by the Ethics Committee of Shanghai Public Health Clinical Center under the number NCT04261517 demonstrated that the prognosis of COVID-19 patients with moderate cases is good. However, a large sample size study is needed to investigate the effects of HCQ in the treatment of COVID-19 (68). A new study is ongoing and can be found on ClinicalTrials.gov under the identifier NCT04303507 with the official title "Chloroquine/Hydroxychloroquine Prevention of Coronavirus Disease (COVID-19)." This study is a double-blinded, randomized, and placebo-controlled trial that will be conducted in a healthcare setting. A total of 40,000 participants will be recruited, and the investigators predict an average of 400–800 participants per site at 50–100 sites. However, the estimated completion date is April 2021.

Anticoagulant Treatments

Since recent findings revealed that most COVID-19 patients with severe cases admitted to the intensive care unit for respiratory failure present predominantly with hypercoagulation, anticoagulant drugs could a potentially prevent a state that could lead to arterial and venous thromboembolic complications (69). Antithrombin and activated protein C for the treatment of classical acute respiratory distress syndrome can be used as anticoagulants for inflammatory thrombus prevention. Platelets may be involved in systemic and local thrombotic responses. Antiplatelet therapies may present a new therapeutic approach. This is a known phenomenon in acute coronary syndromes, where anticoagulant therapy along with antiplatelet therapy decreases arterial thrombosis, but it is associated with an increase in bleeding risk (42).

Therapies Targeting Viral Replication

Remdesivir is an antiviral molecule with a chemical formula of C27H35N6O8P. Remdesivir prevents viral replication by inhibiting viral DNA polymerase. Its antiviral activity has been demonstrated against Ebola virus in multiple human cell types, including primary macrophages and human endothelial cells, with low half-maximal effective concentration (EC_{50}) values of 0.06–0.14 μM (33). It has also been shown that remdesivir inhibits SARS-CoV in primary human airway epithelial cell cultures, which are a biologically relevant *in vitro* model of pulmonary infection (70). Moreover, remdesivir has exhibited antiviral activity against the Marburg virus (33). SARS-CoV and SARS-CoV-2 present 82% RNA sequence homology, and their RNA-dependent RNA polymerase (RdRp) sequences share 96% sequence similarity. Therefore, drugs targeting the viral RdRp proteins of SARS-CoV are also suspected to be effective against SARS-CoV-2. According to the *in vitro* antiviral activity of remdesivir, the *in vivo* tests showed the suppression of Ebola virus replication and the protection of all infected animals against lethal infection (33). In addition, remdesivir decreased the viral load in the lungs and preserved the pulmonary function of mice during SARS-CoV infection (70). These findings suggest that remdesivir can be used as a potential new therapeutic approach for human infections caused by coronaviruses, including SARS-CoV-2. In fact, the first case of COVID-19 in Washington, USA, was treated with intravenous remdesivir. During the treatment, no obvious adverse effects were observed (71). However, we cannot comment yet on the efficiency of the treatment effect of remdesivir during the COVID-19 outbreak.

There are four clinical trials currently enrolling patients in the United States. Moreover, two clinical trials in China have been registered on ClinicalTrials.gov: NCT04257656 for severe disease and NCT04252664 for mild-to-moderate disease (72). Recently, Yeming et al. published the results of the NCT04257656 clinical trial, which showed no clear outcome because of the death or discharge of patients (73). Moreover, in another clinical trial, the benefit in terms of the time to clinical improvement was not statistically significant (21 vs. 23 days), even though the study was underpowered (74). There are limited safety data for remdesivir, which should be obtained in further studies.

Therapies Targeting Viral Transcription

Ribavirin is a broad-spectrum nucleoside antiviral drug that is phosphorylated in virus-infected cells. Basically, the entry of the product into virus-infected cells leads to its phosphorylation. This product acts as a competitive inhibitor of the viral synthetase, interfering with early viral transcription events and thereby hindering the synthesis of ribonucleoproteins and subsequent viral spread. Several controversial *in vitro* studies investigating ribavirin have been conducted. While a few of them have demonstrated that ribavirin has an antiviral effect on SARS, others have revealed no evidence of its antiviral role (75, 76). Additionally, a clinical trial reported no significant antiviral effects on SARS-infected patients (77). In fact, the same study reported side effects, such as haemolytic anemia, resulting from the clinical administration of ribavirin (77). During the COVID-19 pandemic, ribavirin combined with interferon was used based on the Chinese treatment guidelines.

BCG Vaccine

The Bacillus Calmette-Guerin (BCG) vaccine against tuberculosis has been demonstrated to reduce mortality during other infections. The protective mechanism involved in tuberculosis infection has been explored *in vivo*. It was demonstrated that BCG vaccination increased IFN-γ production by CD4+ cells (78). T cells play a crucial role in viral infections; CD4 T cells provide B cell help for antibody production and control the response of other immune cell subsets, whereas CD8 T cells kill infected cells to reduce the viral burden. To better understand the role of T cell responses in SARS-CoV-2 infection, some studies are beginning to be conducted. During SARS-CoV-1 infection, the occurrence of lymphopenia with

drastically reduced numbers of both CD4 and CD8 T cells in moderate and severe COVID-19 cases has been described in several current reports (79). Th1 and Th17 cells play a crucial role in the induction of CD4+ and CD8+ memory cells that are involved in the control of the immune system response during non-mycobacterial secondary infections. Interestingly, BCG vaccination continued to increase Th1 and Th17 responses at least 1 year after vaccination in healthy subjects (80). COVID-19 infection severity is associated with a sharp decrease in the frequency of CD4+ and CD8+ cells and the expression of INF-γ on the surface of CD4+ cells (78). The nonspecific effects of the BCG vaccine present a potential therapeutic method to increase memory responses and enhance the immune system during viral infections that might aid in combating the COVID-19 pandemic.

DISCUSSION

Improved understanding of the viral entry mechanisms and the inflammatory response generated during infection would allow the development of appropriate therapeutic strategies to manage patients with COVID-19. The different therapeutic strategies (**Table 1**) discussed in this review are encouraging and have been proposed to treat or prevent the spread of COVID-19. In addition, most of the described compounds are readily available, and they are known to result in a minor risk of adverse events. Several clinical trials are in process to validate the results. However, these strategies are not without risks, and special attention to factors such as age, sex, and associations with other chronic diseases must be considered during patient selection. Non-specific proinflammatory cytokine targeting during COVID-19 treatment using corticoids, e.g., may favor viral spread. However, targeting specific individual cytokines does not increase viral infection and prevents cytokine storm inflammation-mediated tissue injury, notably in the lung. Since observations have indicated that there are two stages of disease, the first of which is characterized by virus spread and the second by the hyperproinflammatory response responsible for respiratory distress, the timing of the initiation of therapy needs to be carefully defined.

In this review, we also mentioned that virus-neutralizing MAbs represent a therapeutic method with a high potential to prevent viral spread. However, the use of immunoglobulin class G (IgG) MAbs may contribute to an ADE mechanism favoring the spread of the virus during treatment. In parallel, these antibodies can also induce anaphylactic shock that is mediated by the FcγR receptor; FcγRIIA is expressed by neutrophils and platelets, in particular (81). These side effects remain poorly studied. The development of IgG4 or $F(ab)'_2$ antibodies to neutralize the virus or to target proinflammatory antibodies that cannot interact with FcγR may prevent this risk.

Finally, the treatment duration should be well-defined in terms of the half-life of molecules to prevent liver toxicity and the immunosuppressive effect.

Thus, the use of monotherapy or combinatorial therapeutic strategies during different stages of COVID-19 infection represent a potential therapeutic strategy to stop the ongoing pandemic.

AUTHOR CONTRIBUTIONS

SB and AB wrote this review. Both authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by grants from INSERM, ANR JC (ANR-17-CE17-0002-01), and DIM1 HEALTH action financée par la Région Ile-de France.

REFERENCES

1. Corman VM, Lienau J, Witzenrath M. [Coronaviruses as the cause of respiratory infections]. *Internist (Berl).* (2019) 60:1136–45. doi: 10.1007/s00108-019-00671-5
2. Fehr AR, Channappanavar R, Perlman S. Middle east respiratory syndrome: emergence of a pathogenic human coronavirus. *Annu Rev Med.* (2017) 68:387–99. doi: 10.1146/annurev-med-051215-031152
3. Hoffmann M, Kleine-Weber H, Schroeder S, Kruger N, Herrler T, Erichsen S, et al. SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor. *Cell.* (2020) 181:271–80.e8. doi: 10.1016/j.cell.2020.02.052
4. Lau JT, Yang X, Pang E, Tsui HY, Wong E, Wing YK. SARS-related perceptions in Hong Kong. *Emerg Infect Dis.* (2005) 11:417–24. doi: 10.3201/eid1103.040675
5. Guan Y, Zheng BJ, He YQ, Liu XL, Zhuang ZX, Cheung CL, et al. Isolation and characterization of viruses related to the SARS coronavirus from animals in southern China. *Science.* (2003) 302:276–8. doi: 10.1126/science.1087139
6. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet.* (2020) 395:497–506. doi: 10.1016/S0140-6736(20)30183-5
7. Chan JF, Yuan S, Kok KH, To KK, Chu H, Yang J, et al. A familial cluster of pneumonia associated with the 2019 novel coronavirus indicating person-to-person transmission: a study of a family cluster. *Lancet.* (2020) 395:514–23. doi: 10.1016/S0140-6736(20)30154-9
8. Zhu F, Cao Y, Xu S, Zhou M. Reply to Comments on 'Co-infection of SARS-CoV-2 and HIV in a patient in Wuhan city, China'. *J Med Virol.* (2020) doi: 10.1002/jmv.25838. [Epub ahead of print].
9. Matsuyama S, Nagata N, Shirato K, Kawase M, Takeda M, Taguchi F. Efficient activation of the severe acute respiratory syndrome coronavirus spike protein by the transmembrane protease TMPRSS2. *J Virol.* (2010) 84:12658–64. doi: 10.1128/JVI.01542-10
10. Shulla A, Heald-Sargent T, Subramanya G, Zhao J, Perlman S, Gallagher T. A transmembrane serine protease is linked to the severe acute respiratory syndrome coronavirus receptor and activates virus entry. *J Virol.* (2011) 85:873–82. doi: 10.1128/JVI.02062-10
11. Imai Y, Kuba K, Penninger JM. Angiotensin-converting enzyme 2 in acute respiratory distress syndrome. *Cell Mol Life Sci.* (2007) 64:2006–12. doi: 10.1007/s00018-007-6228-6
12. Skeggs LT, Dorer FE, Levine M, Lentz KE, Kahn JR. The biochemistry of the renin-angiotensin system. *Adv Exp Med Biol.* (1980) 130:1–27. doi: 10.1007/978-1-4615-9173-3_1
13. Corvol P, Williams TA, Soubrier F. Peptidyl dipeptidase a: angiotensin I-converting enzyme. *Methods Enzymol.* (1995) 248:283–305. doi: 10.1016/0076-6879(95)48020-X
14. Tschope C, Schultheiss HP, Walther T. Multiple interactions between the renin-angiotensin and the kallikrein-kinin systems: role of ACE inhibition

and AT1 receptor blockade. *J Cardiovasc Pharmacol.* (2002) 39:478–87. doi: 10.1097/00005344-200204000-00003
15. Li F, Li W, Farzan M, Harrison SC. Structure of SARS coronavirus spike receptor-binding domain complexed with receptor. *Science.* (2005) 309:1864–8. doi: 10.1126/science.1116480
16. Gu Y, Hsu AC, Pang Z, Pan H, Zuo X, Wang G, et al. Role of the innate cytokine storm induced by the influenza A virus. *Viral Immunol.* (2019) 32:244–51. doi: 10.1089/vim.2019.0032
17. England JT, Abdulla A, Biggs CM, Lee AYY, Hay KA, Hoiland RL, et al. Weathering the COVID-19 storm: lessons from hematologic cytokine syndromes. *Blood Rev.* (2020) 2020:100707. doi: 10.1016/j.blre.2020.100707
18. Yao X, Ye F, Zhang M, Cui C, Huang B, Niu P, et al. In vitro antiviral activity and projection of optimized dosing design of hydroxychloroquine for the treatment of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). *Clin Infect Dis.* (2020) ciaa237. doi: 10.1093/cid/ciaa237. [Epub ahead of print].
19. Wang M, Cao R, Zhang L, Yang X, Liu J, Xu M, et al. Remdesivir and chloroquine effectively inhibit the recently emerged novel coronavirus (2019-nCoV) in vitro. *Cell Res.* (2020) 30:269–71. doi: 10.1038/s41422-020-0282-0
20. Wu R, Wang L, Kuo HD, Shannar A, Peter R, Chou PJ, et al. An update on current therapeutic drugs treating COVID-19. *Curr Pharmacol Rep*. (2020) 2020:1–15. doi: 10.1007/s40495-020-00216-7
21. Silva JC, Mariz HA, Rocha LF, Jr., Oliveira PS, Dantas AT, et al. Hydroxychloroquine decreases Th17-related cytokines in systemic lupus erythematosus and rheumatoid arthritis patients. *Clinics (São Paulo).* (2013) 68:766–71. doi: 10.6061/clinics/2013(06)07
22. Ohkoshi M, Fujii S. Effect of the synthetic protease inhibitor [N,N-dimethylcarbamoyl-methyl 4-(4-guanidinobenzoyloxy)-phenylacetate] methanesulfate on carcinogenesis by 3-methylcholanthrene in mouse skin. *J Natl Cancer Inst.* (1983) 71:1053–7.
23. Ikeda S, Manabe M, Muramatsu T, Takamori K, Ogawa H. Protease inhibitor therapy for recessive dystrophic epidermolysis bullosa. In vitro effect and clinical trial with camostat mesylate. *J Am Acad Dermatol.* (1988) 18:1246–52. doi: 10.1016/S0190-9622(88)70130-9
24. Sai JK, Suyama M, Kubokawa Y, Matsumura Y, Inami K, Watanabe S. Efficacy of camostat mesilate against dyspepsia associated with non-alcoholic mild pancreatic disease. *J Gastroenterol.* (2010) 45:335–41. doi: 10.1007/s00535-009-0148-1
25. Yamamoto M, Matsuyama S, Li X, Takeda M, Kawaguchi Y, Inoue JI, et al. Identification of nafamostat as a potent inhibitor of middle east respiratory syndrome coronavirus s protein-mediated membrane fusion using the split-protein-based cell-cell fusion assay. *Antimicrob Agents Chemother.* (2016) 60:6532–9. doi: 10.1128/AAC.01043-16
26. Iwaki M, Ino Y, Motoyoshi A, Ozeki M, Sato T, Kurumi M, et al. Pharmacological studies of FUT-175, nafamostat mesilate. V. Effects on the pancreatic enzymes and experimental acute pancreatitis in rats. *Jpn J Pharmacol.* (1986) 41:155–62. doi: 10.1254/jjp.41.155
27. Berry JD, Hay K, Rini JM, Yu M, Wang L, Plummer FA, et al. Neutralizing epitopes of the SARS-CoV S-protein cluster independent of repertoire, antigen structure or mAb technology. *MAbs.* (2010) 2:53–66. doi: 10.4161/mabs.2.1.10788
28. ter Meulen J, van den Brink EN, Poon LL, Marissen WE, Leung CS, Cox F, et al. Human monoclonal antibody combination against SARS coronavirus: synergy and coverage of escape mutants. *PLoS Med.* (2006) 3:e237. doi: 10.1371/journal.pmed.0030237
29. Ng OW, Keng CT, Leung CS, Peiris JS, Poon LL, Tan YJ. Substitution at aspartic acid 1128 in the SARS coronavirus spike glycoprotein mediates escape from a S2 domain-targeting neutralizing monoclonal antibody. *PLoS ONE.* (2014) 9:e102415. doi: 10.1371/journal.pone.0102415
30. Lip KM, Shen S, Yang X, Keng CT, Zhang A, Oh HL, et al. Monoclonal antibodies targeting the HR2 domain and the region immediately upstream of the HR2 of the S protein neutralize in vitro infection of severe acute respiratory syndrome coronavirus. *J Virol.* (2006) 80:941–50. doi: 10.1128/JVI.80.2.941-950.2006
31. ter Meulen J, Bakker AB, van den Brink EN, Weverling GJ, Martina BE, Haagmans BL, et al. Human monoclonal antibody as prophylaxis for SARS coronavirus infection in ferrets. *Lancet.* (2004) 363:2139–41. doi: 10.1016/S0140-6736(04)16506-9
32. van den Brink EN, Ter Meulen J, Cox F, Jongeneelen MA, Thijsse A, Throsby M, et al. Molecular and biological characterization of human monoclonal antibodies binding to the spike and nucleocapsid proteins of severe acute respiratory syndrome coronavirus. *J Virol.* (2005) 79:1635–44. doi: 10.1128/JVI.79.3.1635-1644.2005
33. Warren TK, Jordan R, Lo MK, Ray AS, Mackman RL, Soloveva V, et al. Therapeutic efficacy of the small molecule GS-5734 against Ebola virus in rhesus monkeys. *Nature.* (2016) 531:381–5. doi: 10.1038/nature17180
34. Agostini ML, Andres EL, Sims AC, Graham RL, Sheahan TP, Lu X, et al. Coronavirus susceptibility to the antiviral remdesivir (GS-5734) is mediated by the viral polymerase and the proofreading exoribonuclease. *mBio.* (2018) 9:e00221-18. doi: 10.1128/mBio.00221-18
35. Lim J, Jeon S, Shin HY, Kim MJ, Seong YM, Lee WJ, et al. Case of the index patient who caused tertiary transmission of COVID-19 infection in korea: the application of lopinavir/ritonavir for the treatment of COVID-19 infected pneumonia monitored by quantitative RT-PCR. *J Korean Med Sci.* (2020) 35:e79. doi: 10.3346/jkms.2020.35.e89
36. Pecheur EI, Borisevich V, Halfmann P, Morrey JD, Smee DF, Prichard M, et al. The synthetic antiviral drug arbidol inhibits globally prevalent pathogenic viruses. *J Virol.* (2016) 90:3086–92. doi: 10.1128/JVI.02077-15
37. Conti P, Ronconi G, Caraffa A, Gallenga CE, Ross R, Frydas I, et al. Induction of pro-inflammatory cytokines (IL-1 and IL-6) and lung inflammation by Coronavirus-19 (COVI-19 or SARS-CoV-2): anti-inflammatory strategies. *J Biol Regul Homeost Agents.* (2020) 34:1. doi: 10.23812/CONTI-E
38. Chen N, Zhou M, Dong X, Qu J, Gong F, Han Y, et al. Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study. *Lancet.* (2020) 395:507–13. doi: 10.1016/S0140-6736(20)30211-7
39. Gautret P, Lagier JC, Parola P, Hoang VT, Meddeb L, Mailhe M, et al. Hydroxychloroquine and azithromycin as a treatment of COVID-19: results of an open-label non-randomized clinical trial. *Int J Antimicrob Agents.* (2020) 2020:105949. doi: 10.1016/j.ijantimicag.2020.105949
40. Molina JM, Delaugerre C, Le Goff J, Mela-Lima B, Ponscarme D, Goldwirt L, et al. No evidence of rapid antiviral clearance or clinical benefit with the combination of hydroxychloroquine and azithromycin in patients with severe COVID-19 infection. *Med Mal Infect.* (2020) 50:384. doi: 10.1016/j.medmal.2020.03.006
41. Russell CD, Millar JE, Baillie JK. Clinical evidence does not support corticosteroid treatment for 2019-nCoV lung injury. *Lancet.* (2020) 395:473–5. doi: 10.1016/S0140-6736(20)30317-2
42. Iba T, Levy JH, Levi M, Connors JM, Thachil J. Coagulopathy of coronavirus disease 2019. *Crit Care Med.* (2020) doi: 10.1097/CCM.0000000000004458
43. Liu Y, Sun W, Guo Y, Chen L, Zhang L, Zhao S, et al. Association between platelet parameters and mortality in coronavirus disease 2019: retrospective cohort study. *Platelets.* (2020) 31:490–6. doi: 10.1080/09537104.2020.1754383
44. Shanmugaraj B, Siriwattananon K, Wangkanont K, Phoolcharoen W. Perspectives on monoclonal antibody therapy as potential therapeutic intervention for Coronavirus disease-19 (COVID-19). *Asian Pac J Allergy Immunol.* (2020) 38:10–8. doi: 10.12932/AP-200220-0773
45. Sui J, Li W, Roberts A, Matthews LJ, Murakami A, Vogel L, et al. Evaluation of human monoclonal antibody 80R for immunoprophylaxis of severe acute respiratory syndrome by an animal study, epitope mapping, and analysis of spike variants. *J Virol.* (2005) 79:5900–6. doi: 10.1128/JVI.79.10.5900-5906.2005
46. Wan Y, Shang J, Graham R, Baric RS, Li F. Receptor recognition by the novel coronavirus from wuhan: an analysis based on decade-long structural studies of SARS coronavirus. *J Virol.* (2020) 94:e00127-20. doi: 10.1128/JVI.00127-20
47. Li JL, Li G, Jing XZ, Li YF, Ye QY, Jia HH, et al. Assessment of clinical sepsis-associated biomarkers in a septic mouse model. *J Int Med Res.* (2018) 46:2410–22. doi: 10.1177/0300060518764717
48. Tanaka T, Narazaki M, Kishimoto T. Immunotherapeutic implications of IL-6 blockade for cytokine storm. *Immunotherapy.* (2016) 8:959–70. doi: 10.2217/imt-2016-0020
49. Pedersen SF, Ho YC. SARS-CoV-2: a storm is raging. *J Clin Invest.* (2020) 130:2202–5. doi: 10.1172/JCI137647
50. Izumiyama T, Mori Y, Mori S, Mori N, Kodama T, Itoi E. The effect of anti-IL-6 receptor antibody for the treatment of McH-lpr/lpr-RA1 mice

that spontaneously developed destructive arthritis and enthesitis. *BMC Musculoskelet Disord.* (2019) 20:286. doi: 10.1186/s12891-019-2664-3
51. Michot JM, Albiges L, Chaput N, Saada V, Pommeret F, Griscelli F, et al. Tocilizumab, an anti-IL6 receptor antibody, to treat Covid-19-related respiratory failure: a case report. *Ann Oncol.* (2020) 31:961–4. doi: 10.1016/j.annonc.2020.03.300
52. Velazquez-Salinas L, Verdugo-Rodriguez A, Rodriguez LL, Borca MV. The role of interleukin 6 during viral infections. *Front Microbiol.* (2019) 10:1057. doi: 10.3389/fmicb.2019.01057
53. Atal S, Fatima Z. IL-6 inhibitors in the treatment of serious COVID-19: a promising therapy? *Pharmaceut Med.* (2020) 1–9. doi: 10.1007/s40290-020-00342-z. [Epub ahead of print].
54. Feldmann M, Maini RN, Woody JN, Holgate ST, Winter G, Rowland M, et al. Trials of anti-tumour necrosis factor therapy for COVID-19 are urgently needed. *Lancet.* (2020) 395:1407–9. doi: 10.1016/S0140-6736(20)30858-8
55. Serbina NV, Pamer EG. Monocyte emigration from bone marrow during bacterial infection requires signals mediated by chemokine receptor CCR2. *Nat Immunol.* (2006) 7:311–7. doi: 10.1038/ni1309
56. Krafts K, Hempelmann E, Skorska-Stania A. From methylene blue to chloroquine: a brief review of the development of an antimalarial therapy. *Parasitol Res.* (2012) 111:1–6. doi: 10.1007/s00436-012-2886-x
57. Lamoureux F, Zoubeidi A. Dual inhibition of autophagy and the AKT pathway in prostate cancer. *Autophagy.* (2013) 9:1119–20. doi: 10.4161/auto.24921
58. Li G, De Clercq E. Therapeutic options for the 2019 novel coronavirus (2019-nCoV). *Nat Rev Drug Discov.* (2020) 19:149–50. doi: 10.1038/d41573-020-00016-0
59. Mizushima N, Yoshimori T, Levine B. Methods in mammalian autophagy research. *Cell.* (2010) 140:313–26. doi: 10.1016/j.cell.2010.01.028
60. Solomon VR, Lee H. Chloroquine and its analogs: a new promise of an old drug for effective and safe cancer therapies. *Eur J Pharmacol.* (2009) 625:220–33. doi: 10.1016/j.ejphar.2009.06.063
61. Savarino A, Di Trani L, Donatelli I, Cauda R, Cassone A. New insights into the antiviral effects of chloroquine. *Lancet Infect Dis.* (2006) 6:67–9. doi: 10.1016/S1473-3099(06)70361-9
62. Vincent MJ, Bergeron E, Benjannet S, Erickson BR, Rollin PE, Ksiazek TG, et al. Chloroquine is a potent inhibitor of SARS coronavirus infection and spread. *Virol J.* (2005) 2:69. doi: 10.1186/1743-422X-2-69
63. van den Borne BE, Dijkmans BA, de Rooij HH, le Cessie S, Verweij CL. Chloroquine and hydroxychloroquine equally affect tumor necrosis factor-alpha, interleukin 6, and interferon-gamma production by peripheral blood mononuclear cells. *J Rheumatol.* (1997) 24:55–60.
64. Kuznik A, Bencina M, Svajger U, Jeras M, Rozman B, Jerala R. Mechanism of endosomal TLR inhibition by antimalarial drugs and imidazoquinolines. *J Immunol.* (2011) 186:4794–804. doi: 10.4049/jimmunol.1000702
65. Coelho AS, Chagas CEP, de Padua RM, Pianetti GA, Fernandes C. A comprehensive stability-indicating HPLC method for determination of chloroquine in active pharmaceutical ingredient and tablets: Identification of oxidation impurities. *J Pharm Biomed Anal.* (2017) 145:248–54. doi: 10.1016/j.jpba.2017.06.023
66. Melles RB, Marmor MF. The risk of toxic retinopathy in patients on long-term hydroxychloroquine therapy. *JAMA Ophthalmol.* (2014) 132:1453–60. doi: 10.1001/jamaophthalmol.2014.3459
67. Zhao X, Jiang Y, Zhao Y, Xi H, Liu C, Qu F, et al. Analysis of the susceptibility to COVID-19 in pregnancy and recommendations on potential drug screening. *Eur J Clin Microbiol Infect Dis.* (2020) 39:1209–20. doi: 10.1007/s10096-020-03897-6
68. Chen J, Liu D, Liu L, Liu P, Xu Q, Xia L, et al. [A pilot study of hydroxychloroquine in treatment of patients with moderate COVID-19]. *Zhejiang Da Xue Xue Bao Yi Xue Ban.* (2020) 49:215–9.
69. Spiezia L, Boscolo A, Poletto F, Cerruti L, Tiberio I, Campello E, et al. COVID-19-related severe hypercoagulability in patients admitted to intensive care unit for acute respiratory failure. *Thromb Haemost.* (2020) 120:998–1000. doi: 10.1055/s-0040-1710018
70. Sheahan TP, Sims AC, Graham RL, Menachery VD, Gralinski LE, Case JB, et al. Broad-spectrum antiviral GS-5734 inhibits both epidemic and zoonotic coronaviruses. *Sci Transl Med.* (2017) 9:396. doi: 10.1126/scitranslmed.aal3653
71. Ko WC, Rolain JM, Lee NY, Chen PL, Huang CT, Lee PI, et al. Arguments in favour of remdesivir for treating SARS-CoV-2 infections. *Int J Antimicrob Agents.* (2020) 2020:105933. doi: 10.1016/j.ijantimicag.2020.105933
72. Bhimraj A, Morgan RL, Shumaker AH, Lavergne V, Baden L, Cheng VC, et al. Infectious diseases society of america guidelines on the treatment and management of patients with COVID-19. *Clin Infect Dis.* (2020) doi: 10.1093/cid/ciaa478. [Epub ahead of print].
73. Wang Y, Zhou F, Zhang D, Zhao J, Du R, Hu Y, et al. Evaluation of the efficacy and safety of intravenous remdesivir in adult patients with severe COVID-19: study protocol for a phase 3 randomized, double-blind, placebo-controlled, multicentre trial. *Trials.* (2020) 21:422. doi: 10.1186/s13063-020-04352-9
74. Davies M, Osborne V, Lane S, Roy D, Dhanda S, Evans A, et al. Remdesivir in treatment of COVID-19: a systematic benefit-risk assessment. *Drug Saf.* (2020) 43:645–56. doi: 10.1007/s40264-020-00966-9
75. Chen F, Chan KH, Jiang Y, Kao RY, Lu HT, Fan KW, et al. In vitro susceptibility of 10 clinical isolates of SARS coronavirus to selected antiviral compounds. *J Clin Virol.* (2004) 31:69–75. doi: 10.1016/j.jcv.2004.03.003
76. Morgenstern B, Michaelis M, Baer PC, Doerr HW, Cinatl J Jr. Ribavirin and interferon-beta synergistically inhibit SARS-associated coronavirus replication in animal and human cell lines. *Biochem Biophys Res Commun.* (2005). 326:905–8. doi: 10.1016/j.bbrc.2004.11.128
77. Booth CM, Matukas LM, Tomlinson GA, Rachlis AR, Rose DB, Dwosh HA, et al. Clinical features and short-term outcomes of 144 patients with SARS in the greater Toronto area. *JAMA.* (2003) 289:2801–9. doi: 10.1001/jama.289.21.JOC30885
78. Mathurin KS, Martens GW, Kornfeld H, Welsh RM. CD4 T-cell-mediated heterologous immunity between mycobacteria and poxviruses. *J Virol.* (2009) 83:3528–39. doi: 10.1128/JVI.02393-08
79. Diao B, Wang C, Tan Y, Chen X, Liu Y, Ning L, et al. Reduction and functional exhaustion of t cells in patients with coronavirus disease 2019 (COVID-19*). Front Immunol.* (2020). 11:827. doi: 10.3389/fimmu.2020.00827
80. Kleinnijenhuis J, Quintin J, Preijers F, Joosten LA, Jacobs C, Xavier RJ, et al. BCG-induced trained immunity in NK cells: Role for non-specific protection to infection. *Clin Immunol.* (2014) 155:213–9. doi: 10.1016/j.clim.2014.10.005
81. Jonsson F, de Chaisemartin L, Granger V, Gouel-Cheron A, Gillis CM, Zhu Q, et al. An IgG-induced neutrophil activation pathway contributes to human drug-induced anaphylaxis. *Sci Transl Med.* (2019) 11:eaat1479. doi: 10.1126/scitranslmed.aat1479

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 21 July 2020
doi: 10.3389/fimmu.2020.01817

The Long Road Toward COVID-19 Herd Immunity: Vaccine Platform Technologies and Mass Immunization Strategies

Lea Skak Filtenborg Frederiksen[1], *Yibang Zhang*[1,2], *Camilla Foged*[1] *and Aneesh Thakur*[1]*

[1] Department of Pharmacy, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark,
[2] Department of Pharmaceutics, School of Pharmacy, Jiangsu University, Zhenjiang, China

There is an urgent need for effective countermeasures against the current emergence and accelerating expansion of coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Induction of herd immunity by mass vaccination has been a very successful strategy for preventing the spread of many infectious diseases, hence protecting the most vulnerable population groups unable to develop immunity, for example individuals with immunodeficiencies or a weakened immune system due to underlying medical or debilitating conditions. Therefore, vaccination represents one of the most promising counter-pandemic measures to COVID-19. However, to date, no licensed vaccine exists, neither for SARS-CoV-2 nor for the closely related SARS-CoV or Middle East respiratory syndrome-CoV. In addition, a few vaccine candidates have only recently entered human clinical trials, which hampers the progress in tackling COVID-19 infection. Here, we discuss potential prophylactic interventions for SARS-CoV-2 with a focus on the challenges existing for vaccine development, and we review pre-clinical progress and ongoing human clinical trials of COVID-19 vaccine candidates. Although COVID-19 vaccine development is currently accelerated via so-called fast-track programs, vaccines may not be timely available to have an impact on the first wave of the ongoing COVID-19 pandemic. Nevertheless, COVID-19 vaccines will be essential in the future for reducing morbidity and mortality and inducing herd immunity, if SARS-CoV-2 becomes established in the population like for example influenza virus.

Keywords: coronavirus, SARS-CoV-2, COVID-19, vaccine, immunopathology, immune response, animal models, herd immunity

OPEN ACCESS

Edited by:
Denise L. Doolan,
James Cook University, Australia

Reviewed by:
Arun Kumar,
Coalition for Epidemic Preparedness Innovations (CEPI), Norway
Thorsten Demberg,
Marker Therapeutics, United States

***Correspondence:**
Aneesh Thakur
aneesh.thakur@sund.ku.dk

Specialty section:
This article was submitted to Vaccines and Molecular Therapeutics, a section of the journal Frontiers in Immunology

Received: *09 May 2020*
Accepted: *07 July 2020*
Published: *21 July 2020*

Citation:
Frederiksen LSF, Zhang Y, Foged C and Thakur A (2020) The Long Road Toward COVID-19 Herd Immunity: Vaccine Platform Technologies and Mass Immunization Strategies. Front. Immunol. 11:1817. doi: 10.3389/fimmu.2020.01817

INTRODUCTION

Coronavirus disease 2019 (COVID-19), caused by the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), emerged in December 2019 in Wuhan, China, and rapidly spread globally due to high transmissibility and pathogenicity (1, 2). According to the World Health Organization (WHO), the disease has infected more than 9.0 million people across 216 countries and territories as of June 23rd 2020, with evidence of ongoing local transmission (3). In most cases, the symptoms of COVID-19 are mild and include fever, cough, and shortness of breath. However,

in certain cases, the disease develops into severe pneumonia and multiple organ failure, primarily in elderly and patients with other underlying diseases or conditions, and it has a mortality rate of ∼3.7% (4). On January 30th 2020, WHO declared COVID-19, a public health emergency of international concern. At present, the understanding of the pathogenesis of and immunity against COVID-19 is incomplete, and there is no approved therapy or prophylaxis against the disease. Hence, there is an urgent need to develop both new therapeutics and prophylactics to contain SARS-CoV-2, given the pandemic spread and the associated enormous global humanitarian and economic losses.

Vaccines represent one of the most successful and cost-effective health interventions in human history (5). According to the WHO, global vaccination programs save up to 2–3 million lives each year by priming the immune system to protect the host against potential pathogens, who would otherwise significantly challenge global health and economy (6). Besides providing individual protection, vaccination programs also aim for so-called *population* or *herd immunity*, i.e., immunization of a large proportion of the population to protect the non-vaccinated, immunologically naïve, and immunocompromised individuals by reducing the percentage of vulnerable hosts to a level below the transmission threshold (7). For example, a global immunization coverage of more than 80% against smallpox virus has reduced the transmission rates to uninfected individuals to such low levels that the virus has been eradicated (6). For measles, 91–94% of a population must be vaccinated to achieve herd immunity and prevent new measles outbreaks (8). Likewise, a threshold of 80–85% is now the target for global eradication of poliovirus (6). These examples illustrate well that the threshold for vaccination-induced herd immunity is pathogen specific. A threshold value of ∼67% is estimated to be sufficient for achieving herd immunity against SARS-CoV-2, assuming that the basic reproductive number (R_0) of the virus is three, i.e., one infected individual infects three new individuals (9). Based on this estimate, ∼5.3 billion vaccine doses are required for a single-dose vaccine, or possibly 12–16 billion in case of a multi-dose vaccine. Therefore, it is clear that inducing herd immunity by mass vaccination would be an incredibly powerful tool to contain the COVID-19 pandemic, but it also represent a massive challenge.

The urgent need for safe and efficacious vaccines against COVID-19 has accelerated the development of a number of vaccine candidates, of which a few have already progressed into phase I/II clinical testing. Globally, academic partners are collaborating with vaccine manufacturers to exploit a number of different novel and established vaccine development and manufacturing platforms in the design of COVID-19 vaccines at an unprecedented pace. Here, we review these global efforts with focus on the vaccine candidates in preclinical and clinical development. We also describe the characteristics of the SARS-CoV-2 virus and the immunopathology of the infection, and discuss the host immune response and animal models.

CHARACTERISTICS OF SARS-COV-2

Genome and Virion

Coronaviruses (CoVs) constitute a genus in the *Coronaviridae* family, which are pleomorphic enveloped viruses (10). The *Coronaviridae* are classified into four subgroups, including (i) alpha (α), (ii) beta (β), (iii) gamma (γ), and (iv) delta (δ) coronaviruses. The former two subtypes usually infect mammals, whereas the latter two subtypes predominantly infect birds. The novel SARS-CoV-2 is a member of the β subgroup, along with SARS-CoV and Middle East respiratory syndrome (MERS)-CoV (11, 12). All CoVs are enveloped, positive single-stranded RNA viruses, and they have relatively large RNA genomes ranging from 26 to 32 kilobases (kb) (12). The genome of SARS-CoV-2 contains a 5′ cap structure and a 3′ poly(A) tail, allowing it to serve as messenger RNA (mRNA) for translation of the replicase polyproteins (**Figure 1A**). The open reading frames (ORFs) 1a/b occupy two-thirds of the genome (∼20 kb) and encode the replicase polyproteins. The replicase polyproteins include the 1–16 non-structural proteins (nsps1-16), which are responsible for (i) viral replication, (ii) RNA-dependent RNA-polymerase activity, (iii) helicase activity, and (iv) assembly of virus replication structures (11). The majority of the remaining one-third of the genome encodes structural and accessory proteins (11–13). Coronaviruses contain four major structural proteins, i.e., the spike (S), envelope (E), membrane (M), and nucleocapsid (N) proteins (**Figure 1B**). The 5′ end of the genome contains a leader sequence and an untranslated region (UTR), including structures required for RNA replication and transcription. The 3′ UTR also encodes RNA structures required for replication and synthesis of viral RNA. The genomic sequence of CoV is 5′-leader-UTR-replicase-S-E-M-N-3′-UTR-poly(A) tail with accessory genes interspersed between the structural proteins at the 3' end of the genome (13). Interestingly, the accessory genes encoding the ORF3b, ORF6, and N proteins are interferon (IFN) antagonists, which act on the type I IFN pathway, either by inhibiting transcription or by acting on effector mechanisms, and they modulate the host innate immune response (14, 15). Like other coronaviruses, SARS-CoV-2 virions are spherical in shape with a diameter of 65–125 nm (16), and the most prominent features include the spikes projections emanating from the surface of the virions. These spike projections give the virus the resemblance of a crown, hence the name coronavirus (12, 17). The S protein represents the *key* on the virion, which binds by *locking* into its receptor on a host cell. The N proteins hold the RNA genome, and together, the S, E, and M proteins constitute the viral envelope (18).

It is crucial to investigate the impact of mutations in the major antigenic proteins of SARS-CoV-2 when developing vaccines and vaccination strategies against SARS-CoV-2. The S protein is the most commonly used SARS-CoV-2 virus protein for vaccine development (19). Recently, 149 mutation sites have been identified across the genome from 103 sequenced strains of SARS-CoV-2 (20), indicating that there is a high mutation rate within these strains. SARS-CoV-2 strains in this study had evolved into two different subtypes (L, which is a more aggressive

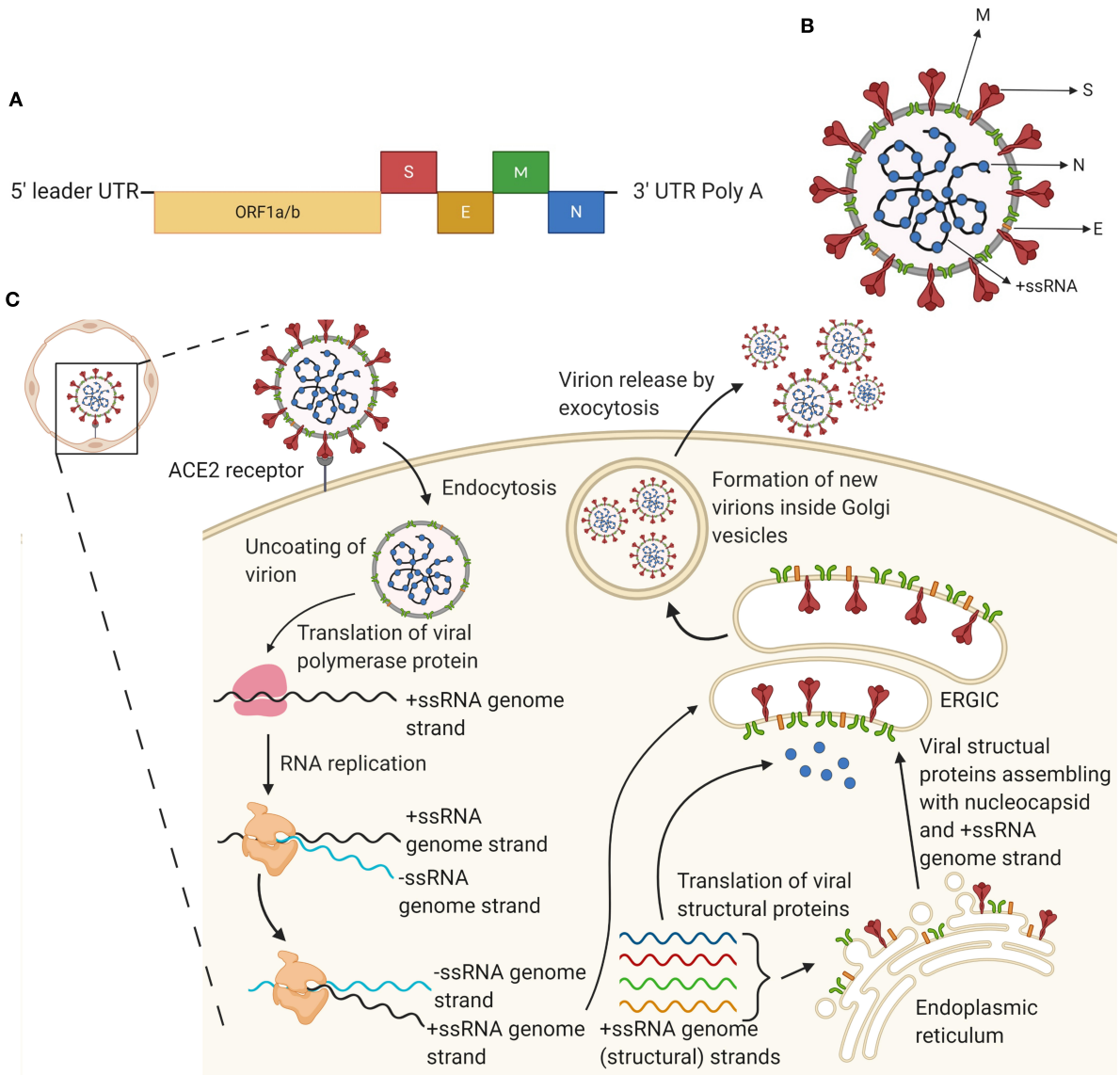


FIGURE 1 | The genome, virion, and replication of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). **(A)** Schematic diagram of the SARS-CoV-2 genome. Approximately two-thirds of the positive single stranded RNA genome encodes a large polyprotein (ORF1a/b; nude). The last third of the genome proximal to the 3′-end encodes four structural proteins, i.e., the spike (S), envelope (E), membrane (M), and nucleocapsid (N) proteins (red, orange, green, and blue, respectively). The colors of the structural proteins are consistent in this figure. **(B)** Schematic diagram of the SARS-CoV-2 virion. The virion displays a nucleocapsid composed of genomic RNA (+ssRNA) and N protein, which is enclosed inside the virus envelope consisting of S, E, and M proteins. **(C)** Schematic overview of the life cycle of SARS-CoV-2 in host cells. The life cycle is initiated upon binding of the S protein to angiotensin-converting enzyme 2 (ACE2) on host cells, e.g., epithelial cells in the alveoli. After receptor binding, a conformational change in the S protein facilitates viral endocytosis and envelope fusion with the cell membrane. Subsequently, viral genomic RNA is released into the host cell, and viral +ssRNA is translated into viral polymerase encoded by the genome, which initiates replication of +ssRNA to –ssRNA and further produces a series of genomic and subgenomic mRNAs. These are translated into viral proteins, which are subsequently assembled with genomic RNA into virions in the endoplasmic reticulum (ER) and the ER-Golgi intermediate compartment (ERGIC) to form mature virions that are trafficked via Golgi vesicles out of the cell by exocytosis. Created with Biorender.com.

type and S, which represents a less aggressive type) with great differences in geographical distribution, transmission ability, and severity of disease (20). Hence, these differences also complicate vaccine design (20). In another study, of the 144 sequences of global SARS-CoV-2 strains, two subtypes SARS-CoV-2a (China strains) and SARS-CoV-2b (USA strains) were identified, which differ only by a novel synonymous mutation of position D614G in the S protein and display different antigenicity (21). Domains containing this mutation point have been confirmed to represent B-cell epitopes (21). Further, it has been reported that the antigenic indexes were reduced more for SARS-CoV-2b than for SARS-CoV-2a (21). These results indicate that different

subtypes may display different antigenicity and that vaccine development may benefit from a strategy focused on targeting multiple subunits of the virus (21).

Viral Replication

SARS-CoV receptor recognition and attachment is initiated via interactions between the S protein and the human angiotensin-converting enzyme 2 (ACE2) expressed by cells in (i) vascular endothelia, (ii) renal and cardiovascular tissue, (iii) epithelia of the airways, small intestine, and testes, and (iv) lung parenchyma [(11, 13); **Figure 1C**]. The S protein of SARS-CoV-2 has been shown to engage with a comparable affinity with human ACE2 as the SARS-CoV S protein (16). Due to the genomic resemblance between the novel SARS-CoV-2 and SARS-CoV, SARS-CoV-2 is expected to display a pathogenesis, which is similar to that of SARS-CoV. ACE2 is suggested to play a protective role in inflamed lung tissue, and the binding of the SARS-CoV S protein to ACE2 is assumed to contribute to disease severity (11). Following receptor binding and attachment, SARS-CoV-2 gains access to the host cell cytosol. This is accomplished by cleavage of the S protein by cathepsin, transmembrane protease serine 2 (TMPRRS2) or another protease, followed by fusion of the viral and cellular membranes (12, 13). The S protein of SARS-CoV-2 has been shown to contain a furin cleavage site between the two polypeptides referred to as the S_1 and S_2 subunits, which is not present in the S protein of SARS-CoV (16). An additional cleavage of the S_2' subunits is important for separating the receptor-binding domain (RBD) and the fusion domains, and for exposing the fusion peptide (13, 16). Subsequently, the fusion peptide is inserted into the membrane, followed by the formation of an antiparallel six-helix bundle, which allows mixing of cellular and viral membranes, eventually resulting in fusion and release of the viral genome into the cytosol (13). The next step for SARS-CoV replication is translation of the replicase gene from the virion genomic RNA. The replicase gene encodes two large ORFs, i.e., *rep1a* and *rep1b*, which code for the co-terminal polyproteins pp1a and pp1b, respectively (13). These polyproteins are subsequently cleaved into nsps1-16, which assemble into the replicase-transcriptase complex, where RNA synthesis takes place. Ultimately, nsps1-16 facilitate RNA replication and transcription of the sub-genomic RNAs (11, 13). Viral RNA synthesis follows the translation and assembly of viral replicase complexes. Both genomic and subgenomic RNAs are produced by viral RNA synthesis through negative-strand intermediates (12, 13). Subgenomic RNAs serve as mRNAs for the structural and accessory genes. After replication and subgenomic RNA synthesis, the structural proteins S, E, and M are translated and inserted into the endoplasmic reticulum (ER) (12). Here, they are transported into the ER-Golgi intermediate compartment (ERGIC), where viral genomes become encapsulated by the N protein, resulting in the formation of mature virions (13). The virions are subsequently transported in vesicles to the cell surface and released through exocytosis, thereby contributing to the generation of new virions able to infect host cells and promote human-to-human transmission (11, 12).

COVID-19 DISEASE

Transmission

According to the WHO, SARS-CoV-2 has killed more than 469,159 and infected over 8,974,795 individuals globally by June 23rd 2020. Hence, SARS-CoV-2 has a higher transmission rate compared to SARS-CoV in 2002–2003, which infected 8,098 and killed more than 700 individuals. This may be the result of genetic recombination in the RBD of the S protein, thus enhancing the transmission ability of SARS-CoV-2 (12). For preventive strategies against SARS-CoV-2, it is important to determine the source of origin and transmission of the virus. The outbreak arose at the Huanan Seafood market in the city of Wuhan, China, and SARS-CoV-2 rapidly infected more than 50 individuals. At this market, which is now closed, live animals were frequently sold, e.g., bats, birds, frogs, rabbits, and snakes. Genomic analyses revealed similarities between SARS-CoV-2 and SARS-like bat viruses, hence bats are suspected to be reservoirs for SARS-CoV-2 (1). In another study, the origin of SARS-CoV-2 has been associated with Pangolin-CoV, because Pangolin-CoV was found to be 91.02 and 90.55% identical to SARS-CoV-2 and Bat-CoV, respectively (22). Close contact with these infected animal reservoirs is the major cause of animal-to-human SARS-CoV-2 transmission (23), which eventually leads to a rapid human-to-human transmission (12, 24). Respiratory droplets and contact transmission are considered as the main transmission routes for human-to-human transmission, and aerosol spread is suspected to be another important transmission route (18). The stability of SARS-CoV-2 on various surfaces has been investigated, indicating that aerosol and fomite transmission of SARS-CoV-2 is plausible, because the virus remains viable and infectious in aerosols for several hours and even up to days on surfaces (25). Pharyngeal virus shedding and active virus replication in the upper respiratory tract has been confirmed (26). Together, these findings stress the importance of good hand hygiene and the use of surgical masks as mitigation strategies against respiratory droplets to prevent SARS-CoV-2 transmission (27). Reports also indicate that SARS-CoV-2 may follow alternative transmission routes (28, 29). Studies have shown a prolonged presence of SARS-CoV-2 viral RNA in fecal samples from infected patients. Urine and rectal swabs from children and adults have also been tested positive, even after negative nasopharyngeal tests, implying a risk of fecal-oral transmission (28, 29).

Clinical Presentation

Typical clinical symptoms of COVID-19 disease include fever, dry cough, dyspnea, headache, and pneumonia. The clinical features revealed by chest computed tomography (CT) present as pneumonia, however abnormal features, e.g., alveolar damage, acute respiratory distress syndrome (ARDS), acute cardiac injury, and incidence of ground-glass opacities have also been reported (1, 30). The symptoms of COVID-19 infection appear after an incubation period of ∼5.2 days (31). The period from the onset of symptoms until death ranges from 6 to 41 days with a median of 14 days (32), depending on the age, immune system status, and care of the patient, and it has been shown to be shorter for patients above 70 years of age (32). The CT findings

and COVID-19 symptoms show similarities to infection with other betacoronaviruses, i.e., SARS-CoV and MERS-CoV. In addition, COVID-19 patients develop gastrointestinal symptoms like diarrhea, emphasizing the importance of testing fecal and urine samples to exclude any potential alternative transmission route (28, 33). A recent review by the Chinese Center for Disease and Prevention including 72,314 cases of COVID-19 showed that <1% of the cases represented children younger than 10 years of age (34).

IMMUNOPATHOLOGY AND HOST IMMUNE RESPONSE

Innate Immune Response

Currently, only limited data is available characterizing the innate immune response of patients against SARS-CoV-2. In one study from Wuhan, China, increased total numbers of neutrophils (38%), reduced total numbers of lymphocytes (35%), increased serum IL-6 levels (52%), and increased c-reactive protein levels (84%) were observed for 99 patients (1). In addition, a meta-analysis of six clinical studies conducted in China showed that the neutrophil-to-lymphocyte ratio was significantly increased in patients with severe COVID-19, whereas the lymphocyte-to-C-reactive ratio protein was significantly decreased (35). In a separate study, the numbers of T cells and $CD8^+$ T cells were significantly lower, while the number of NK cells was reduced considerably in patients with severe COVID-19, as compared to the numbers for individuals with mild disease (36). Furthermore, an exuberant increase of the plasma levels of interferon gamma-induced protein 10 (IP-10), monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein 1α (MIP-1α), and tumor necrosis factor alpha (TNF-α) was associated with disease deterioration and a fatal outcome [(37); **Figure 2**]. These clinical features suggest a remarkably higher pro-inflammatory condition in the disease progression and severity than previously reported for SARS-CoV and MERS-CoV infection, suggesting a potential cytokine storm-mediated disease severity [(38); **Figure 2**].

Like SARS-CoV, SARS-CoV-2 exploits the receptor ACE2 to gain entry into cells. ACE2 is widely expressed in cardiopulmonary tissues and in hematopoietic cells, including monocytes and macrophages (38). To mount an antiviral response, innate immune cells recognize virus invasion by pathogen-associated molecular patterns (PAMPs), which in the case of RNA viruses is either viral genomic ssRNA or double-stranded RNA. This genomic RNA is recognized either by endosomal RNA receptors, including Toll-like receptor (TLR)-3 and TLR7, or by the cytosolic retinoid-inducible gene (RIG)/melanoma differentiation-associated gene 5 (MDA5) receptor (39). Following recognition, a downstream signaling cascade is activated, which in turn activates a number of transcription factors, i.e., nuclear factor κB (NF-κB), activator protein 1 (AP-1), IFN response factor 3 (IRF3), and IRF7, which is accompanied by their translocation into the nucleus. These transcription factors induce the expression of type I IFN (IFN-α and IFN-β) and pro-inflammatory cytokines, e.g., TNF and IL-1, and chemokines, e.g., C–C motif chemokine ligand 2 and C-X-C motif chemokine ligand 8, which comprise the first line of anti-viral immune defense (39). The binding of IFN to the IFNα/β receptor activates the Janus kinase-signal transducer and activator of transcription 1 (JAK-STAT) pathway, which brings the receptor-associated kinases JAK1 and Tyk2 into close proximity, eventually resulting in phosphorylation of STAT1 and STAT2. STAT1/2 form complexes with IFN regulatory factor 9 (IRF9), which subsequently translocate into the nucleus to initiate transcription of IFN-stimulated genes (ISGs) (39). Induction of a type I IFN response may be sufficient to inhibit viral replication and dissemination in the early stage of viral infection (40). However, the production of type I IFN (IFN-α and IFN-β), which constitute key antiviral mediators, is inhibited in COVID-19 patients (41, 42). Reportedly, coronaviruses have evolved several immune evasion mechanisms to restrict the early induction of type I IFN (43, 44).

Adaptive Immune Response

Neutralizing antibodies (nAbs) induced by virus infection play a crucial role in controlling viral infection. For SARS-CoV-2, nAbs limit the infection at a later phase and prevent re-infection upon a future encounter with the virus (45). Recently developed SARS-CoV- and MERS-CoV-specific nAbs target the S1-RBD, S1- N-terminal domain (NTD) and S2 region, respectively, and block protein-receptor interaction and interfere with viral entry into the host cell, hence inhibiting viral infection (46). However, no SARS-CoV-2-specific nAbs have been reported so far. SARS-CoV nAbs with potential cross-reactivity and/or cross-neutralizing activity against SARS-CoV-2 infection are currently being identified (45) because SARS-CoV-2 is closely related to SARS-CoV, and the S proteins of the two different viruses display high sequence identity (1). Encouragingly, recent studies show that nAbs from convalescent SARS patients can block SARS-CoV-2 from entering target cells *in vitro*, which suggests potential cross-protective epitopes between the two viruses (1, 47).

T cell-mediated immune responses in SARS-CoV have been well-elucidated (48). Both $CD4^+$ and $CD8^+$ T-cells provide broad and long-term protection against coronavirus infections. $CD4^+$ T cells promote the proliferation of virus-specific antibodies by activating T-cell dependent B cells, whereas $CD8^+$ T cells are cytotoxic and kill virus-infected cells. In COVID-19 patients, a significant T cytopoenia was observed in circulating $CD4^+$ and $CD8^+$ T cells (49). Furthermore, a progressive increase in the PD-1^+CD8^+ and Tim-3^+CD8^+ subpopulation, which corresponds to exhausted T cells, was observed in symptomatic patients (49). In another study, the function of NK and $CD8^+$ T cells was exhausted with the increased expression of natural killer group 2 member A (NKG2A) in COVID-19 patients (36). Recently, the decreased T cell proportion in patients with severe COVID-19 was associated with a down-regulated gene expression related to Th17 cell activation and differentiation (50). In one study investigating samples from convalescent SARS-CoV infected patients, a higher magnitude of $CD8^+$ T cells, as compared to $CD4^+$ T cells, was observed. Both $CD4^+$ and $CD8^+$ T cells from patients with severe disease

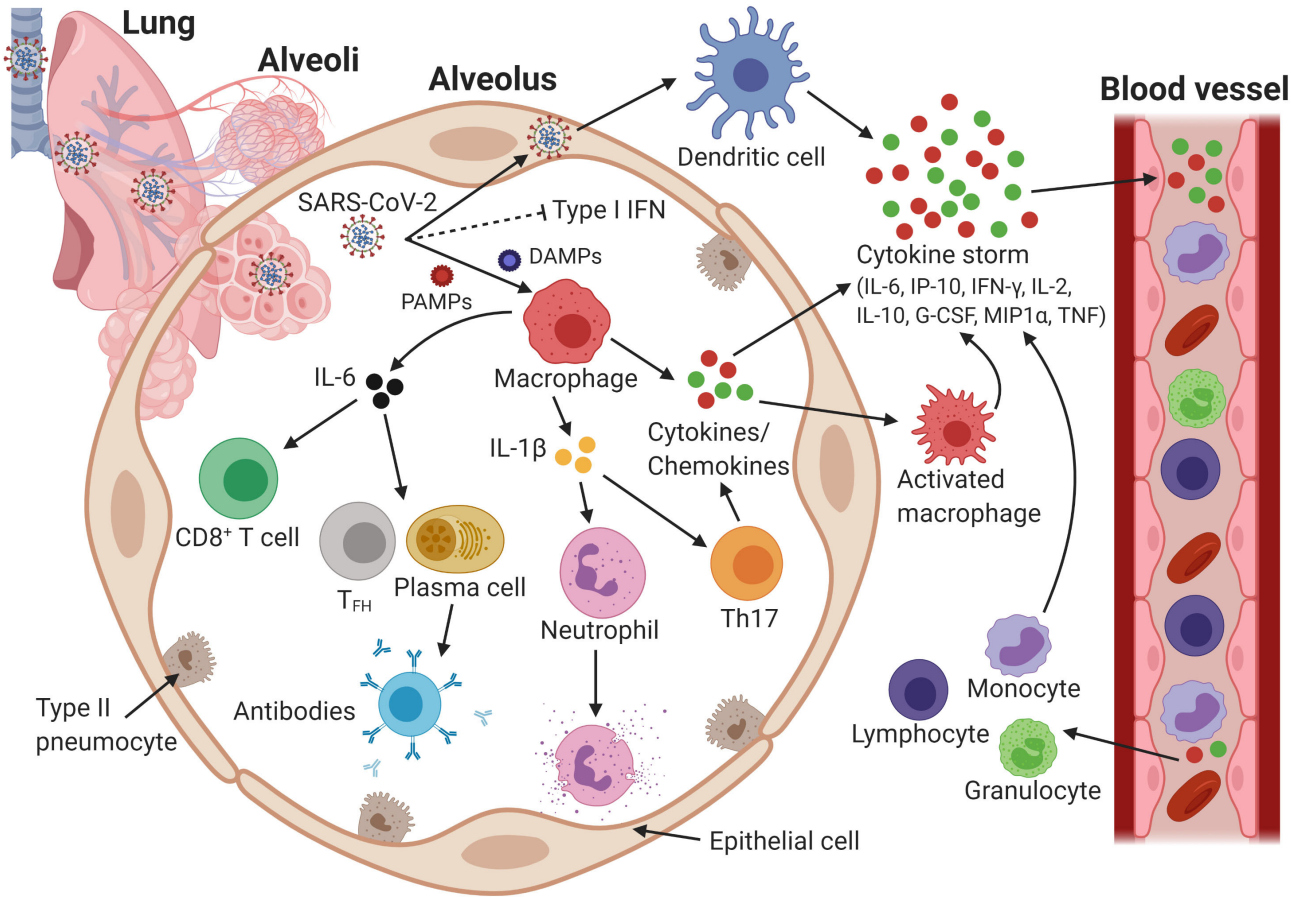


FIGURE 2 | Host immune response and immunopathology during severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection. SARS-CoV-2 infects cells expressing the surface receptors angiotensin- converting enzyme 2 (ACE2) and transmembrane serine protease 2 (TMPRSS2). SARS-CoV-2 dampens anti-viral type I IFN responses, which results in uncontrolled viral replication. Viral pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) activate epithelial cells, endothelial cells, and tissue-resident macrophages to release proinflammatory cytokines and chemokines, including interleukin 6 (IL-6), IFN gamma-induced protein 10 (IP-10), IFN gamma (IFN-γ), IL-2, IL-10, macrophage inflammatory protein 1α (MIP1α), MIP1β, monocyte chemoattractant protein 1 (MCP1), granulocyte colony-stimulating factor (G-CSF), and tumor necrosis factor alpha (TNF-α). Cytokine- and chemokine-activated macrophages and virus-infected dendritic cells mediate extensive production of additional cytokines and chemokines, which eventually initiates a so-called cytokine storm. Chemokines attract more inflammatory cells that migrate from the blood vessels into the lungs, and these cells intensify the cytokine storm by releasing additional proinflammatory chemokines and cytokines, hence establishing a proinflammatory feedback loop. The cytokines circulate to other organs via the blood, eventually causing multi-organ damage. The downstream production of the cytokines IL-6 and IL-1β recruits neutrophils and $CD8^+$ T cells, which not only control viral growth but also induce tissue damage, leading to alveolar flooding and consolidation (acute respiratory distress syndrome). IL-6 may recruit T-helper type 17 cells (Th17), which exacerbate inflammatory responses following activation. IL-6 also recruits follicular helper T cells (T_{FH}) and B cells/plasma cells, which produce SARS-CoV-2-specific antibodies that may help virus neutralization. Alternatively, B cells produce non-neutralizing antibodies that enhance SARS-CoV-2 infection through antibody-dependent enhancement, which further exacerbate organ damage. Created with Biorender.com.

displayed a central memory phenotype, as compared to the cells from patients with mild disease. Strong T-cell responses correlated with high titers of nAbs, while a Th2 type cytokine response (IL-4, IL-5, and IL-10) was detected in patients with a fatal outcome (51). The strong evidence that a Th1 type immune response plays a significant role in clearing SARS-CoV and MERS-CoV infection applies presumably also for clearance of SARS-CoV-2 infection. A recent study reported SARS-CoV-2-specific $CD4^+$ T cells in all and $CD8^+$ T cell responses in most COVID-19 patients (52). Importantly, this study also identified SARS-CoV-2-reactive $CD4^+$ T cells in ∼40–60% of unexposed individuals, which suggests cross-reactive T cell recognition between circulating "common cold" coronaviruses and SARS-CoV-2.

ANIMAL MODELS

Validated and predictive animal models represent important tools in the translation of vaccine candidates from bench to bedside because they help improving the understanding of disease biology and the requirements for developing of safe and efficacious vaccines. Validation of animal models is based on the criteria that animal models represent humans in terms of (i) comparable disease biology and clinical symptoms,

i.e., face validity, (ii) displaying clinical interventions, which exhibit similar biological effect, i.e., predictive validity, and (iii) analogous function of the therapeutic target, i.e., target validity (53). An ideal animal model is immunocompetent and reproduces the typical features of human disease as closely as possible upon receiving a bio-relevant dose of challenge virus via an appropriate inoculation route (54).

Models based on mice, which are easy to breed and handle, often represent the animal models of choice in biomedical research, and murine models would be relevant for COVID-19 vaccine research. However, wild-type mice are resistant to SARS-CoV-2 infection because murine ACE2 is significantly different from the human receptor (1). However, genetically modified heterozygous mice that express both the murine and the human ACE2 receptor have been developed and used for testing of novel vaccine candidates during the SARS-CoV outbreak (55). Compared to wild-type mice that display only mild symptoms, transgenic mice expressing the human ACE2 receptor develop clinical illness after SARS-CoV-2 infection, including weight loss and interstitial pneumonia, and viral antigens have been detected in their bronchial epithelial cells, alveolar macrophages, and alveolar epithelial cells (56). However, the expression of the human ACE2 receptor in transgenic mice is not physiological, and transgenic mice are currently not readily available for testing of SARS-CoV-2 vaccine candidates. ACE2 knockout mice have been used in ARDS and SARS research and may also be useful for studying ARDS associated with COVID-19 (57). Transmembrane serine protease 2 (TMPRSS2) knockout mice may also be useful for investigating COVID-19 pathogenesis because TMPRSS2 is involved in cellular SARS-CoV-2 entry (58). In addition, STAT1 knockout mice develop progressive lung disease, including diffuse interstitial pneumonia and spread to other systemic organs, hence they may be useful for studying disease pathogenesis (1, 58). Adaptation of SARS-CoV by serial passage in the lungs of BALB/c mice resulted in a virus (MA15) that was lethal for young mice following intranasal inoculation and was preceded by high viral titer in the lungs, viremia, and spreading of virus to other systemic organs (59). With the availability of mouse-adapted SARS-CoV-2 isolates, it is expected that inbred mice could be useful to study the disease and evaluate novel vaccine candidates and antiviral drugs (60). Young inbred mice, for example of the strains BALB/c, C57BL/6, and 129S6, support SARS-CoV replication, but without development of disease, and these strains may be useful for evaluating immune responses to COVID-19 infection and vaccines (61). On the other hand, old (12–14 months) BALB/c mice exhibit patchy interstitial pneumonia following SARS-CoV infection, hence they can be used for COVID-19 research, especially to model the age-related higher mortality in humans (62). Aged C57BL/6 and 129S6 mice can also be used for these studies, but they exhibit lower viremia, as compared to BALB/c mice, following SARS infection (63). C57BL/6 mice have been used in SARS (64) and MERS (65) coronavirus-induced ARDS and can also be used for studying ARDS associated with COVID-19.

Ferrets have been widely used as a model for studying several respiratory viruses (66, 67). Viral replication has been detected both in the upper and lower respiratory tract of ferrets after infection with influenza and SARS-CoV (66–68). However, SARS-CoV-2 was found to replicate only in the nasal turbinate, soft palate, and tonsils of ferrets (69). SARS-CoV-2 can apparently also replicate in the digestive tract of ferrets, because viral RNA has been detected in the rectal swabs, but the virus was not detected in the lung lobes of ferrets, even after intratracheal inoculation (69). Between ferrets and humans, there is a difference of two amino acids in the segment of ACE2 to which SARS-CoV-2 first attaches (69), but the reason for the inability of SARS-CoV-2 to replicate in the lower respiratory tract of ferrets remains elusive. Despite this, the replication of SARS-CoV-2 in the ferret upper respiratory tract implies that ferrets represents an interesting animal model for evaluation of COVID-19 vaccine candidates.

Golden Syrian hamster is another widely used experimental animal model, which supports replication of SARS-CoV (63, 70) but not MERS-CoV, which uses the dipeptidyl peptidase−4 protein for viral entry (71). Golden Syrian hamsters represent a suitable experimental animal model for SARS-CoV−2 infection, because efficient viral replication takes place in the upper and lower respiratory epithelial cells, the animals display apparent clinical signs accompanied with weight loss, and high viral titers are found in the lungs and the intestine (72). Moreover, SARS-CoV-2 infection in Golden Syrian hamsters not only satisfies the Koch's postulates [(i) the pathogen must be present in every case of the disease, (ii) the pathogen must be isolated from the diseased host and grown in pure culture, (iii) the specific disease must be reproduced when a pure culture of the pathogen is inoculated into a healthy susceptible host, and (iv) the pathogen must be recoverable from the experimentally infected host] but also indicates virus transmission between challenged hamsters and naïve contact hamsters housed in the same cages (72). The differences in the susceptibility of mice and hamsters to SARS-CoV-2 infection are suggested to be related to the fact that in mice, 11 of the 29 amino acids present in the SARS-CoV-2 spike-contacting regions of ACE2 differ in the human ACE2 as compared to only four amino acids in hamster ACE2 (72). Nevertheless, in contrast to the large animal models and ACE2-transgenic mice, the Golden Syrian hamster model is easily available, physiologically relevant, and closely reflects COVID-19 infection, hence it represents a useful tool for studying the pathogenesis, treatment, and vaccines for COVID-19.

Despite being expensive, not readily available, and difficult to handle, non-human primates (NHPs) often represent the last stage of animal testing before any drug or vaccine candidate can enter clinical trials. NHPs are the gatekeepers for clinical trials due to their close genetic relationship with humans. Among the NHPs, African greens, rhesus macaques, cynomolgus macaques, and marmosets are being studied for SARS-CoV-2 infection. In one study including eight cynomolgus macaques, four of the oldest macaques excreted virus from the nose and the throat without any clinical signs after SARS-CoV-2 infection (73). The virus was detected in type I and II pneumocytes and in ciliated epithelial cells of the nasal, bronchial, and bronchiolar mucosa (73). In another study, two rhesus macaques that recovered from SARS-CoV-2 infection were reinfected after confirmed recovery, but they did not shown any signs of COVID-19 4 weeks later

(74). This finding suggests a possible protection following natural infection or vaccination against COVID-19. In another study, older rhesus macaques infected with SARS-CoV-2 exhibited more severe interstitial pneumonia than younger macaques (75). This age-related difference in the pathogenicity of SARS-CoV-2 in NHPs may be useful for evaluation of therapeutics and vaccines due to the close correlation to humans. In a recent study, rhesus macaques were rechallenged with SARS-CoV-2 and displayed a 5 $\log_{10}$ reduction in the viral titers in the bronchoalveolar lavage and nasal mucosa, as compared to the primary infection, which suggests that the SARS-CoV-2 infection induces protective immunity against a subsequent exposure (76). Efforts are also underway to develop NHP models that can mimic the co-morbidities in COVID-19, e.g., hypertension and diabetes.

VACCINE PLATFORM TECHNOLOGIES

In the past decades, a wide array of novel vaccine platform technologies has been developed, thanks to advances primarily in molecular biology and vaccinology. These platform technologies range from inactivation and targeted attenuation of live pathogens to the delivery of synthetic peptide antigens and recombinantly produced protein antigens, as well as virus-like particles (VLPs), non-replicating and replicating viral vectors, polysaccharide-protein conjugates, and nucleic acid-based (DNA and RNA) vaccines. The existing marketed vaccines against infectious diseases are based on many of these platform technologies (77, 78). However, it is striking that all types of vaccine platform technologies are currently evaluated against COVID-19 in preclinical animal models (**Figure 3A** and **Table 1**), and some of them have even progressed into clinical development (**Figure 3B** and **Table 2**). This broad diversity increases the chances that at least a few of the candidates eventually will become approved and marketed.

Inactivated Vaccines

Many approved vaccines are so-called inactivated vaccines based on inactivated pathogens, including the vaccines against polio, typhoid, cholera, plague, pertussis, and influenza. A few COVID-19 vaccine candidates based on this well-established technology are evaluated in preclinical studies [(79–81); **Table 1**]. This includes a formalin-inactivated COVID-19 vaccine candidate developed by Osaka University, Japan similar to their previous formalin-inactivated West Nile virus vaccine (79), which was found to be protective in mice and immunogenic in NHPs (80). Researchers at Colorado State University (Fort Collins, CO, USA) are developing an inactivated virus vaccine for COVID-19 (SolaVAX), which is based on an existing technology platform for pathogen inactivation in blood products including the use of ultraviolet light and riboflavin to inactivate the virus by targeted damage of nucleic acids while preserving the integrity of proteins and viral antigens (81, 82). This strategy has been shown to be efficient for inactivating MERS-CoV (83). Sinovac Biotech (Beijing, China) in collaboration with Dynavax (Emeryville, CA, USA) will evaluate the combination of Sinovac's chemically inactivated COVID-19 vaccine candidate (86) with Dynavax's advanced adjuvant CpG 1018 (84, 85). Sinovac is also testing their chemically-inactivated whole SARS-CoV-2 virus particles (PiCoVacc) developed in VERO monkey cells and the adjuvant alum (86) in phase I/II clinical trials (**Table 2**). PiCoVacc induced SARS-CoV-2-specific neutralizing antibodies in mice, rats, and NHPs and conferred complete protection in NHPs against SARS-CoV-2 (86). Using the same platform technology, candidate vaccines against influenza (176) and SARS (177) where shown to be safe and immunogenicity in phase I clinical trials. Wuhan Institute of Biological Products (Wuhan, China) and Beijing Institute of Biological Products (Beijing, China) are testing their vaccine candidates, which have been prepared by growing the SARS-CoV-2 in the VERO monkey cell line and inactivated with chemicals [(19); **Table 2**]. The development of conventional inactivated vaccines requires the cultivation of high titers of infectious virus, which in the case of SARS-CoV-2 has to take place in biosafety level 3 facilities, which is of major safety concern. Moreover, incomplete virus inactivation constitutes a potential risk to vaccine production workers and may also cause disease outbreaks in vaccinated populations and induce harmful immune or inflammatory responses.

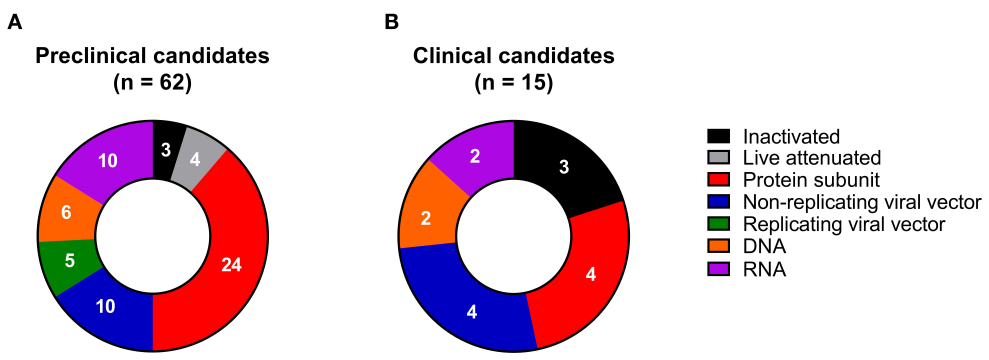


FIGURE 3 | Examples of COVID-19 vaccine candidates in **(A)** preclinical ($n = 62$) and **(B)** clinical development ($n = 15$), grouped according to vaccine platform technology.

TABLE 1 | Examples of COVID-19 vaccine candidates in preclinical development.

Vaccine platform	Vaccine candidate/Information	Developer	Status	Trial/Production site	Link	Reference to the technology
INACTIVATED						
	Formalin-inactivated	Osaka University/BIKEN/National Institutes of Biomedical Innovation, Health and Nutrition (NIBIOHN)	Animal testing planned	Osaka, Japan	WHO	(79, 80)
	SolaVAX: Chemically inactivated	Colorado State University	Animal testing ongoing	Fort Collins, CO, USA	Colorado State University	(81–83)
	Inactivated vaccine + CpG 1018 adjuvant	Sinovac/Dynavax	Animal testing planned	Emeryville, CA, USA; Beijing, China	Dynavax	(84–86)
LIVE ATTENUATED						
	Viral de-optimized live attenuated vaccine	Codagenix/Serum Institute of India	Animal test results from mice and primates in August 2020	Farmingdale, NY, USA	Codagenix	(87, 88)
	Attenuated measles virus	German Center for Infection Research (DZIF)	Animal testing in mice in Autumn 2020	Brunswick, Germany	DZIF	(89, 90)
	Attenuated measles virus	Etna Biotech	Advancing preclinical candidate	Catania, Italy	Zydus Cadila	(91)
	Codon de-optimization technology	Griffith University/Indian Immunologicals	Ongoing animal testing	Brisbane, Australia; Hyderabad, India	Indian Immunologicals	–
PROTEIN SUBUNIT						
	Recombinant vaccine of SARS-CoV-2 S protein expressed in baculovirus system + pandemic adjuvant system (squalene, dl-α-tocopherol and polysorbate 80)	Sanofi Pasteur/GSK/Biomedical Advanced Research and Development Authority (BARDA)	Advancing preclinical candidate; clinical trial to begin between March and August 2021	Lyon, France; Brentford, UK; Washington, DC, USA	Sanofi Pasteur	(92–95)
	Molecular clamp-stabilized S protein	University of Queensland/GSK/CSIRO/ Viroclinics Xplore	Clinical testing in July, 2020	Queensland, Australia; Brentford, UK; Canberra, Australia; Rotterdam, The Netherlands	GSK University of Queensland	https://patentscope.wipo.int/search/en/detail.jsf?docId=WO2018176103; (96)
	COVID-19 XWG-03: truncated S protein	GSK/Xiamen Innovax Biotech Co., Ltd./Xiamen University	Advancing preclinical candidate	Brentford, UK; Xiamen, Fujian, China	GSK	(97–99)
	S protein	AJ Vaccines	Advancing preclinical candidate	Copenhagen, Denmark	AJVaccines	–
	S protein	Walter Reed Army Institute of Research (WRAIR)/U Army Medical Institute of Infectious Diseases	Ongoing animal testing	Maryland, United States	WRAIR	(100, 101)
	S protein	EpiVax/University of Georgia	Advancing preclinical candidate	Providence, RI, USA; Athens, GA, USA	EpiVax	(102–105)
	S protein	VIDO-InterVac, University of Saskatchewan	Ongoing animal testing	Saskatoon, SK, Canada	VIDO-InterVac	(106, 107)
	Adjuvanted S protein	National Institute of Infectious Disease	Advancing preclinical candidate	Tokyo, Japan	Japanese Agency for Medical Research and Development	(108, 109)
	PittCoVacc: Microneedle arrays S1 subunit	University of Pittsburgh	Clinical testing in Summer, 2020	Pittsburgh, PA, USA	University of Pittsburgh	(110)
	Recombinant protein, nanoparticles (based on S-protein and other epitopes)	Saint-Petersburg scientific research institute of vaccines and serums	Clinical testing in 2021	Saint-Petersburg, Russia	WHO	–

(Continued)

TABLE 1 | Continued

Vaccine platform	Vaccine candidate/Information	Developer	Status	Trial/Production site	Link	Reference to the technology
	Heat shock protein gp-96 backbone for multiple antigens	Heat Biologics/University of Miami	Advancing preclinical candidate	Morrisville, NC, USA; Miami, FL, USA	Heat Biologics	(111, 112)
	Receptor-binding domain (RBD) protein	Baylor College of Medicine/Texas Children's Hospital	Advancing preclinical candidate	Houston, TX, USA	Baylor College of Medicine	(113, 114)
	Adjuvanted RBD protein	Biological E Ltd.	Advancing preclinical candidate	Hyderabad, India	WHO	–
	DPX-COVID-19: Oil-based formulation with peptides epitopes of S protein	IMV Inc.	Clinical testing in Summer 2020	Québec, Canada	IMV	(115, 116)
	Human signal peptide domain complexed with undisclosed SARS-CoV-2 protein(s) as vaccine	Vaxil Bio Therapeutics	Advancing preclinical candidate (identified by *in silico* analysis)	Ness Ziona, Israel	Vaxi Bio Therapeutics	(117, 118)
	FlowVax COVID-19: Peptide, dry powder for injection or nasal spray	Flow Pharma Inc.	NHP testing in April 2020	East Palo Alto, CA, USA	Flow Pharma	(119, 120)
	Ii-Key hybrid peptide	Generex/EpiVax	Clinical testing in June, 2020	Toronto, Canada; Providence, RI, USA	EpiVax	(102, 103, 121, 122)
	Adjuvanted microsphere peptide	University of Saskatchewan	Ongoing animal testing	Saskatoon, SK, Canada	University of Saskatchewan	(123, 124)
	Synthetic long peptide vaccine candidate for S and M proteins	OncoGen	Advancing preclinical candidate	Timisoara, Romania	OncoGen	https://www.preprints.org/manuscript/202002.0102/v1
	Recombinant *Lactobacillus acidophilus* expressing S protein	Colorado State University	Advancing preclinical candidate	Fort Collins, CO, USA	Colorado State University	(125, 126)
	Drosophila S2 insect cell expression system virus-like particles (VLPs) (Split-protein conjugation system)	ExpreS^{2}ion/Adaptvac/University of Copenhagen	Clinical testing in April, 2021	Hørsholm, Denmark; Netherlands	ExpreS2ion/Adaptvac	(127–130)
	IBIO-200: Subunit protein (Virus-Like Particle), plant produced	iBio/CC-Pharming	Ongoing animal testing	Bryan, TX, USA; Beijing, China	iBio	(131, 132)
	VLP-recombinant protein administered with an adjuvant	Osaka University/BIKEN/NIBIOHN	Advancing preclinical candidate	Osaka, Japan	WHO	–
NON-REPLICATING VIRAL VECTOR						
	Ad26 (alone or with MVA boost)	Janssen Pharmaceutical Companies (Johnson & Johnson)/BARDA	Clinical testing in September 2020	New Jersey, USA	Johnson & Johnson	(76, 133)
	Modified Vaccinia Ankara encoded virus-like particles (MVA-VLP)	GeoVax/BravoVax	Ongoing animal testing	Atlanta, GA, United States; Wuhan, China	GeoVax	(134, 135)
	MVA-S encoded	DZIF—German Center for Infection Research	Animal testing in mice in Summer 2020	Brunswick, Germany	DZIF	(136, 137)
	AdCOVID: Adenovirus-based NasoVAX expressing SARS2-CoV S protein; nasal spray	Altimmune	Clinical testing in quarter three of 2020	Maryland, USA	Altimmune	https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6253025/pdf/ofy209.162.pdf
	Ad5 S (GREVAX^{TM} platform)	Greffex	Animal testing ongoing	Houston, USA	Greffex	
	SARS-CoV-2 protein VLP produced in tobacco	Medicago Inc.	Clinical testing in Summer 2020	Quebec, Canada	Medicago	(138, 139)

(Continued)

TABLE 1 | Continued

Vaccine platform	Vaccine candidate/Information	Developer	Status	Trial/Production site	Link	Reference to the technology
	Oral recombinant vaccine through adenovirus type 5 vector (Ad5)	Vaxart Inc.	Preclinical; Phase I in second half of 2020	San Francisco, USA	Vaxart	(140, 141)
	Adenovirus VLPs expressing SARS2-CoV S protein	Imophoron/University of Bristol	Animal testing planned	Bristol, UK	Imophoron	(142)
	Adenovirus vector expressing SARS2-CoV S protein	ReiThera/LEUKOCARE/ Univercells	Clinical testing in Summer 2020	Rome, Italy; Munich, Germany; Brussels, Belgium	ReiThera	(143, 144)
	Parainfluenza virus 5 expressing S protein	University of Georgia/University of Iowa	Animal testing ongoing	Athens, GA, USA; Iowa City, IA, USA	University of Georgia	(145)
REPLICATING VIRAL VECTOR						
	Measles vector	Institute Pasteur/Themis/University of Pittsburg Center for Vaccine Research	Animal testing planned	Paris, France; Vienna, Austria; Pittsburgh, PA, USA	Themis	(146, 147)
	TNX-1800: Horsepox vector expressing S protein	Tonix Pharma/Southern Research	Animal testing planned	Birmingham, AL, USA; New York, USA	Tonix Pharma	(148, 149)
	Vesicular stomatitis virus (VSV) vector expressing S protein	International AIDS Vaccine Initiative (IAVI)/Batavia Biosciences	Animal testing ongoing	New York, USA; Leiden, The Netherlands	IAVI	(150, 151)
	Influenza vector expressing RBD	University of Hong Kong	Clinical testing in July 2020	Hong Kong	University of Hong Kong	(152, 153)
	CoroFlu: Influenza virus expressing S protein	University of Wisconsin Madison/ FluGen/Bharat Biotech	Clinical testing in Fall 2020	Madison, WI, United States; Hyderabad, India	University of Wisconsin Madison	(154, 155)
DNA						
	DNA plasmid vaccine (electroporation)	Zydus Cadila	Advancing preclinical candidate	Ahmedabad, India	Zydus Cadila	–
	Four linear DNA-based vaccine candidates	Takis/Applied DNA Sciences/Evvivax	Preclinical testing in Autumn 2020	Stony Brook, USA; Rome, Italy	Evvivax	(156, 157)
	DNA	Osaka University/AnGes/Takara Bio	Animal testing in April 2020	Tokyo, Japan	AnGes	(158, 159)
	DNA with electroporation	Karolinska Institute/Cobra Biologics	Advancing preclinical candidate	Staffordshire, UK; Stockholm, Sweden	Cobra Biologics	(160, 161)
	Plasmid DNA, needle-free delivery	Immunomic Therapeutics, Inc./EpiVax, Inc./PharmaJet, Inc.	Animal testing ongoing	Rockville, MD, USA; Providence, RI, USA; Golden, CL, USA	Immunomix	(102, 103, 162, 163)
	DNA, nasal delivery	University of Waterloo	Advancing preclinical candidate	Waterloo, ON, Canada	University of Waterloo	(164, 165)
RNA						
	RNAoptimizer® technology	CureVac	Clinical testing in June 2020	Tubingen, Germany	CureVac	–
	mRNA	BIOCAD	Animal testing in April 2020	St. Petersburg, Russia	BIOCAD	–
	Lipid nanoparticle (LNP)-encapsulated mRNA	China CDC/Tongji University/Stermirna Therapeutics	Clinical testing in April 2020	Beijing, China	Xinhuanet.com	–
	LNP-encapsulated mRNA cocktail encoding VLP and LNP-encapsulated mRNA encoding RBD	Fudan University, Shanghai JiaoTong University, and RNACure Biopharma	Animal testing ongoing	Shanghai, China	Fudan University	http://chinaxiv.org/abs/202002.00070
	LNP-encapsulated saRNA	Imperial College London	Clinical testing in June 2020	UK	Imperial College London	(166, 167)

(Continued)

TABLE 1 | Continued

Vaccine platform	Vaccine candidate/Information	Developer	Status	Trial/Production site	Link	Reference to the technology
	LNP-encapsulated saRNA	Arcturus Therapeutics/Duke-National University of Singapore	Animal testing ongoing	San Diego, USA; Singapore	Arcturus Therapeutics	(168)
	mRNA for intranasal delivery	eTheRNA Immunotherapies/EpiVax/Nexelis, REPROCELL/Centre for the Evaluation of Vaccination	Clinical testing in early 2021	Niel, Belgium	eTheRNA	(169, 170)
	mRNA	Sanofi Pasteur/Translate Bio	Animal testing planned	Lyon, France; Lexington, MA, United States	Sanofi Pasteur	(171, 172)
	Replication defective SARS-CoV-2 derived RNAs	Centro Nacional Biotecnología (CNB-CSIC)	Advancing preclinical candidate	Madrid, Spain	CNB-CSIC	(173, 174)
	LNP-encapsulated mRNA	University of Tokyo/Daiichi-Sankyo	Advancing preclinical candidate	Tokyo, Japan	Daiichi-Sankyo	(175)

Live Attenuated Vaccines

Live attenuation of pathogens is yet another conventional vaccine technology, which is exploited for at least four novel COVID-19 vaccine candidates (**Table 1**). Live attenuated vaccines against several viruses have been commercialized, including influenza virus, rotavirus, polio virus, yellow fever virus, and measles virus. A live-attenuated vaccine has several advantages, including inducing an immune response against several different antigens of the virus and the possibility for scale-up for mass production. Codagenix (Farmingdale, NY, USA) and the Serum Institute of India (Pune, India) are co-developing a live-attenuated vaccine candidate against SARS-CoV-2 using rational, computer-aided gene design and chemical synthesis through a process referred to as *viral gene deoptimization* (87). A vaccine against respiratory syncytial virus designed using this technique has previously been shown to induce protective immunity in NHPs (88). The German Center for Infection Research (DZIF, Braunschweig, Germany) and Zydus Cadila (Etna Biotech, Ahmedabad, India) are developing a live attenuated recombinant measles virus (rMV) vectored vaccine against COVID-19. Using rMV, Etna Biotech has demonstrated the ability of a live attenuated human papillomavirus virus (HPV) vaccine to induce nAbs in NHPs (91), while DZIF has shown protection against infection with MERS-CoV (89) and Zika virus (90) in mice using this platform. Indian Immunologicals (Hyderabad, India) in collaboration with Griffith University (Brisbane, Australia) is exploiting the codon de-optimization technology to develop a live attenuated COVID-19 vaccine. Although live attenuated vaccines that target respiratory viral infections have been approved for use in humans, the fact that the virus is excreted in the feces of SARS-CoV-2-infected individuals (178, 179) generate concern that a live attenuated SARS-CoV-2 vaccine strain may also be excreted in the feces and can potentially transmit to unvaccinated individuals. Yet another potential matter of concern is the risk of recombination of a live attenuated vaccine virus with wild-type CoV.

Subunit Vaccines

Subunit vaccines are based on synthetic peptide(s) or recombinant protein(s) of the target pathogen. Several approved vaccines are subunit vaccines, for example vaccines against HPV, hepatitis B virus and influenza virus. Unlike inactivated viruses, live attenuated viruses, and virus-vectored vaccines, subunit vaccines only contain specific viral antigenic fragments and do not include any additional components of the pathogenic viruses. Therefore, this approach eliminates the concerns of incomplete viral inactivation, virulence recovery, and pre-existing anti-vector immunity (180). Hence, subunit vaccines are generally considered very safe. In addition, subunit vaccines can specifically target well-characterized neutralizing antigenic epitopes and, in combination with adjuvants, improve immunogenicity, and/or efficacy (180). Because the S protein of SARS-CoV-2 plays a vital role in receptor binding and membrane fusion, vaccines targeting the S protein are suggested to be capable of inducing antibodies that can neutralize virus infection by blocking virus binding and fusion (181). Therefore, the S protein constitutes a major target antigen for SARS-CoV-2 subunit vaccine candidates (**Table 1**). However, in addition to the full-length S protein and its antigenic fragments, the S1 subunit, NTD, RBD, and the S2 subunit may also be important antigen targets for the development of subunit vaccines (20). Sanofi Pasteur (Lyon, France) and GlaxoSmithKline (GSK, London, UK) are developing a COVID-19 subunit vaccine candidate, where Sanofi contributes with an S-protein antigen, which is based on recombinant DNA technology using a baculovirus expression platform (92, 93). Using this platform, Sanofi has licensed a recombinant influenza vaccine in the USA (93). GSK contributes with a pandemic adjuvant technology based

TABLE 2 | COVID-19 vaccine candidates in clinical trials.

Study title	Vaccine	Sponsor	Location	Status	Phase	Primary outcome	Study identifier
Safety and Immunogenicity Study of 2019-nCoV Vaccine (mRNA-1273) to Prevent SARS-CoV-2 Infection; Dose-Confirmation Study to Evaluate the Safety, Reactogenicity, and Immunogenicity of mRNA-1273 COVID-19 Vaccine in Adults Aged 18 Years and Older	mRNA-1273	National Institute of Allergy and Infectious Diseases (NIAID)/Moderna Therapeutics	Washington, USA	Recruiting	I; II	Relevant safety outcomes (12 months follow up); Adverse events (28 days post-vaccination); SARS-CoV-2-specific binding antibody (through 1 year after the final dose)	NCT04283461; NCT04405076
Immunity and Safety of Covid-19 Synthetic Minigene Vaccine	LV-SMENP-DC vaccine and antigen-specific CTLs	Shenzhen Geno-Immune Medical Institute	Guangdong, China	Recruiting	I/II	Clinical improvement based on a 7-point scale (28 days after randomization); Lower Murray lung injury score (7 days after randomization)	NCT04276896
Safety and Immunity of Covid-19 aAPC Vaccine	Pathogen-specific aAPC	Shenzhen Geno-Immune Medical Institute	Guangdong, China	Recruiting	I	Frequency of vaccine events; Frequency of serious vaccine events; Proportion of subjects with positive T cell response	NCT04299724
A Phase I Clinical Trial in 18-60 Adults (APICTH); A Phase II Clinical Trial to Evaluate the Recombinant Vaccine for COVID-19 (Adenovirus Vector) (CTII-nCoV); Phase I/II Clinical Trial of Recombinant Novel Coronavirus Vaccine (Adenovirus Type 5 Vector) in Canada	Recombinant Novel Coronavirus Vaccine (Adenovirus Type 5 Vector)	CanSino Biologics Inc./Institute of Biotechnology, China	Hubei, China; Halifax, Canada	Recruiting/Active, not recruiting; Not yet recruiting	I/II	Adverse reactions 0–7 days post-vaccination. Adverse reactions (0–14 days post-vaccination); IgG and neutralizing antibodies (28 days post-vaccination); Adverse reactions (0–6 and 0–28 days and 6 months after post-vaccination)	NCT04313127/ ChiCTR2000030906; NCT04341389; NCT04398147
A Study of a Candidate COVID-19 Vaccine (COV001) and Investigating a Vaccine Against COVID-19	ChAdOx1 nCoV-19	University of Oxford/Advent Srl	UK	Not yet recruiting	I/II and II/III	Efficacy, safety, and immunogenicity (6 months); Efficacy and safety (6 months)	NCT04324606 and NCT04400838
Evaluating the Safety, Tolerability and Immunogenicity of bacTRL-Spike Vaccine for Prevention of COVID-19	bacTRL-Spike (orally)	Symvivo Corporation	Canada	Not yet recruiting	I	Frequency of adverse events (up to 12 months post-vaccination)	NCT04334980
Safety, Tolerability and Immunogenicity of INO-4800 for COVID-19 in Healthy Volunteers	INO-4800 administered intradermally	Inovio Pharmaceuticals	Missouri and Pennsylvania, USA	Recruiting	I	Adverse events, injection site reactions, antigen-specific binding antibody titers and, IFN-γ responses (baseline up to week 28)	NCT04336410
Safety and Immunogenicity Study of 2019-nCoV Vaccine (Inactivated) for Prophylaxis SARS CoV-2 Infection (COVID-19); Safety and Immunogenicity Study of Inactivated Vaccine for Prevention of SARS-CoV-2 Infection (COVID-19)	Inactivated SARS-CoV-2	Sinovac Biotech Co., Ltd.	Jiangsu, China; Hebei, China	Recruiting; Not yet recruiting	I/II	Safety indexes of adverse reactions; Immunogenicity indexes of neutralizing-antibody seroconversion rates (up to 28 days after the whole schedule vaccination) Seroconversion rates of neutralizing antibody (30th day after the 2nd dose)	NCT04352608; NCT04383574

(Continued)

TABLE 2 | Continued

Study title	Vaccine	Sponsor	Location	Status	Phase	Primary outcome	Study identifier
Study to Describe the Safety, Tolerability, Immunogenicity, and Potential Efficacy of RNA Vaccine Candidates Against COVID-19 in Healthy Adults; A Trial Investigating the Safety and Effects of Four BNT162 Vaccines Against COVID-2019 in Healthy Adults	BNT162 (BNT162a1, BNT162b1, BNT162b2) (Prime/Boost), BNT162c2 (Single Dose)	BioNTech RNA Pharmaceuticals GmbH and Pfizer	Mainz, Germany; Berlin, Germany	Recruiting; Recruiting	I/II	Solicited local reactions at the injection; Solicited systemic reactions (up to 7 ± 1 day after each immunization); Treatment-emergent adverse event (up to 21 ± 2 day after prime immunization and 28 ± 4 days after boost immunization)	NCT04368728; NCT04380701
Evaluation of the Safety and Immunogenicity of a SARS-CoV-2 rS (COVID-19) Nanoparticle Vaccine With/Without Matrix-M Adjuvant	SARS-CoV-2 rS and Matrix-M Adjuvant	Novavax	Victoria and Queensland, Australia	Not yet recruiting	I	Solicited adverse events (28 days); Serum IgG antibody levels specific for the SARS-CoV-2 rS protein antigen(s) (35 days)	NCT04368988
SCB-2019 as COVID-19 Vaccine	SCB-2019 with or without AS03 or CpG 1018 + Alum	Clover Biopharmaceuticals AUS Pty Ltd.	Australia	Not yet recruiting	I	Solicited adverse events (7 days after the first or second vaccination); Antibody Titers (Day 1 to Day 184)	NCT04405908
A clinical study for effectiveness and safety evaluation for recombinant chimeric COVID-19 epitope DC vaccine in the treatment of novel coronavirus pneumonia	Recombinant chimeric COVID-19 epitope DC vaccine	Shenzhen Third People's Hospital	Guangdong, China	Recruiting	I/II	Duration of disease; Antipyretic rate; Severe rate	ChiCTR2000030750
A randomized, double-blinded, placebo-controlled phase II clinical trial for Recombinant Novel Coronavirus (2019-nCOV) Vaccine (Adenovirus Vector)	Adenovirus type 5 vector vaccine	Jiangsu Provincial Center for Disease Control and Prevention	Jiangsu, China	Not yet recruiting	II	Adverse reactions 0–14 days post-vaccination; Anti-SARS-CoV-2 neutralizing antibody titer on day 28 post-vaccination	ChiCTR2000031781
A randomized, double-blind, placebo parallel-controlled phase I/II clinical trial for inactivated Novel Coronavirus Pneumonia vaccine (Vero cells)	Inactivated	Wuhan Institute of Biological Products Co., Ltd.	Wuhan, Hubei, China	Not yet recruiting	I/II	Incidence of adverse reactions/events (up to 7 days); Four-fold growth rate and antibody level, and cellular immunity (up to 90, 180, and 360 days)	ChiCTR2000031809
A phase I/II clinical trial for inactivated novel coronavirus (2019-CoV) vaccine (Vero cells)	Inactivated	Beijing Institute of Biological Products Co., Ltd.	Beijing, China	Recruiting	I/II	Incidence of adverse reactions/events (up to 7 days); Four-fold growth rate and antibody level (up to 28 days); Cellular immunity (Up to 28, 180, and 360 days)	ChiCTR2000032459

aAPC, artificial antigen-presenting cell; COVID-19, coronavirus disease 2019; CTLs, cytotoxic T lymphocytes; DC, dendritic cell; LNP, lipid nanoparticle; mRNA, messenger RNA; nCoV, novel coronavirus; SARS-CoV-2, SARS coronavirus 2; S protein, SARS-CoV-2 spike protein. Sources: Chinese Clinical Trial Register website (www.chictr.org.cn); ClinicalTrials.gov website (www.clinicaltrials.gov); EU Clinical Trials Register (www.clinicaltrialsregister.eu).

on the Adjuvant System 03 (AS03) comprising of squalene, dl-α-tocopherol, and polysorbate 80 (94, 95). GSK is also testing their adjuvant in collaboration with Clover Biopharmaceuticals, University of Queensland (Brisbane, Australia), and Xiamen Innovax Biotech (Xiamen, China). Utilizing its patented Trimer-Tag© technology, Clover Biopharmaceuticals has developed a SARS-CoV-2 S-Trimer subunit vaccine candidate SCB-2019 that resembles the native trimeric viral spike (182, 183). SCB-2019 is in phase I clinical testing with AS03 (94, 95) or CpG 1018 and alum adjuvants [(84, 85); **Table 2**]. Researchers at the University of Queensland are using the patented *molecular clamp* technology, which involves synthesizing a protein and subsequently clamping it onto virus-infected cells (184) as shown previously against flaviviruses (96). Molecular clamp-stabilized

S protein will be combined with GSK adjuvants. COVID-19 XWG-03 is a preclinical vaccine candidate developed by Xiamen University (Xiamen, China) and Xiamen Innovax Biotech using the GSK adjuvant AS04 (monophosphoryl lipid A and aluminum hydroxide) (97). COVID-19 XWG-03 is based on a series of truncated S proteins, which will be screened in combination with AS04. Xiamen Innovax Biotech has previously developed similar *Escherichia coli*-produced subunit vaccines against HPV (98) and hepatitis E (99) in humans. The Walter Reed Army Institute of Research (Silver Spring, MD, USA) is also targeting the S protein and has previously demonstrated efficacy of a MERS-CoV S1-protein subunit vaccine in mice and NHPs (100), as well as in camels and alpacas (101). EpiVax (Providence, RI, USA) is exploiting the proprietary iVAX toolkit that comprises a suite of immunoinformatics algorithms for sorting candidate antigens, selecting immunogenic and conserved T cell epitopes, and eliminating regulatory T-cell epitopes (102–104). The optimized S-protein antigens will be tested for immunogenicity and protection against a SARS-CoV-2 challenge in collaboration with University of Georgia (Athens, GA, USA), which has previously tested the platform against influenza (105). Vaccine and Infectious Disease Organization—International Vaccine Centre (VIDO-InterVac, University of Saskatchewan, Saskatoon, Canada) is developing an S protein subunit vaccine based on prior experience with testing of a MERS-CoV vaccine candidate in NHPs (106, 107). The National Institute of Infectious Disease (Tokyo, Japan) is aiming at developing a new vaccine by combining an undisclosed adjuvant and an antigen using recombinant protein synthesis as previously demonstrated against influenza virus H5N1 (108, 109). PittCoVacc is a subunit vaccine candidate from University of Pittsburgh (Pittsburgh, PA, USA) that is based on a microneedle array (MNA) embedded SARS-CoV-2 S1 protein, which was recently found to elicit strong antigen-specific antibody responses for up to 2 weeks in mice (110). This MNA platform is currently tested in clinical trials against cutaneous T-cell lymphoma (*ClinicalTrials.gov Identifier: NCT02192021*). The COVID-19 vaccine candidate of Heat Biologics (Morrisville, NC, USA) is based on its secreted heat shock protein chaperone gp96 platform and has been shown to induce protection against simian immunodeficiency virus (SIV) in NHPs (111, 112). Vaccine researchers at Baylor College of Medicine and Texas Children's Hospital (both Houston, TX, USA) are using their experience with developing a SARS vaccine antigen consisting of the RBD of the SARS-CoV S protein to develop a similar vaccine against SARS-CoV-2 (113, 114).

In addition to vaccines based on full protein, several vaccine developers are investigating peptides antigens as vaccine candidates against COVID-19. IMV Inc. (Québec, Canada) is developing a vaccine candidate based on the IMV's DPX delivery technology and incorporating peptides targeting S protein epitopes of SARS-Cov-2 as shown previously for respiratory syncytial virus (RSV) (115) and anthrax (116). Vaxil Bio Therapeutics (Toronto, Ontario, Canada) is using the proprietary bioinformatic approach VaxHit™ to identify signal peptide domains of SARS-CoV-2 proteins as shown for mucin 1 tumor-associated antigen in mice (117) and in multiple myeloma patients (118). FlowVax COVID-19 is a candidate vaccine from Flow Pharma (Palo Alto, CA, USA) consisting of an adjuvanted, thermostable, and biodegradable peptide-loaded microsphere vaccine targeting the SARS-CoV-2 nucleocapsid. Flow Pharma has developed and tested a Zika virus vaccine candidate that induces cytotoxic T cell (CTL) responses in mice (119, 120). Generex Biotechnology (Toronto, Canada) is using EpiVax's computational tools to predict epitopes that can be used to generate peptide-based COVID-19 vaccines using the patented NuGenerex Immuno-Oncology Ii-Key technology (NGIO). NGIO technology has been used to develop peptide-based vaccine candidates, which have been tested against a HPV16+ cancer model in mice (121) and prostate cancer in humans (122). University of Saskatchewan's VIDO-InterVac is developing a peptide-based, microsphere-adjuvanted COVID-19 vaccine candidate using a combination adjuvant platform (TriAdj) (123) comprising of a TLR agonist (either polyinosinic-polycytidylic acid or CpG oligodeoxynucleotides), a host defense peptide, and polyphosphazene. TriAdj has been used to generate vaccine-induced protective immunity against several infectious diseases in animals and humans (124, 185). Colorado State University is developing a novel oral COVID-19 vaccine candidate using recombinant *Lactobacillus acidophilus* expressing the viral S protein. This platform has been shown to induce Th1 and Th17 responses against HIV-1 epitopes in mice after oral administration (125, 126). ExpreS^{2}ion Biotechnology (Hørsholm, Denmark), Adaptvac (Hørsholm, Denmark), and University of Copenhagen is applying a *Drosophila melanogaster* Schneider 2 stable cell line expression system expressing VLPs to generate a novel vaccine candidate as previously used for malaria in human clinical trials (127, 128). They are utilizing this split-protein conjugation technology to generate stable isopeptide-bound antigen-VLP complexes by mixing antigen and VLP components. The technology has been demonstrated to induce broadly nAbs specific for HIV-1 V3 glycan in mice and macaques (129), and it has been used to develop a combinatorial HPV and placental malaria vaccine (130). iBio (Newark, DE, USA) in partnership with CC-Pharming (Beijing, China) is working on iBIO-200, which is a COVID-19 candidate vaccine based on Agrobacterium-mediated transient protein production in tobacco (Nicotiana benthamiana) plants and has been used for delivering recombinant proteins into mammalian cells (131) and for generating strong virus-specific nAb responses in animals (132).

Novavax (Gaithersburg, MD, USA) with support from the Coalition for Epidemic Preparedness Innovations (CEPI, Oslo, Norway), is clinically testing their COVID-19 subunit vaccine candidate prepared using the proprietary Sf9/baculovirus recombinant technology platform to generate S protein antigens as done previously for an RSV vaccine candidate (186). The protein antigens have been combined with a saponin-based Matrix-M™ adjuvant [(187); **Table 2**]. A subunit-based vaccine candidate from Shenzhen Third People's Hospital (Guangdong, China) in phase I/II testing is aimed at evaluating the effectiveness and safety of the recombinant chimeric COVID-19 epitope DC vaccine in the treatment of SARS-CoV-2-induced pneumonia.

Non-replicating Viral Vector Vaccines

Viral vectors are used to deliver vaccine antigens to the target cells or tissues. A wide variety of replicating and non-replicating viral vectors are available. Adenoviruses and poxviruses represent examples of viral vectors, of which both replicating and non-replicating forms are available. Vectors designed primarily as replication-defective or non-replicating viral vectors include adeno-associated virus, alphavirus, and herpesvirus, while replicating vectors include measles virus, vesicular stomatitis virus, poliovirus, and yellow fever virus. Several of the non-replicating viral vector-based COVID-19 vaccine candidates in preclinical testing are based on adenovirus vectors (**Table 1**). Janssen (Johnson & Johnson, Leiden, The Netherlands) is using the AdVac® technology (based on adenovirus type 26) alone or in combination with the MVA-BN® technology based on a Modified Vaccinia Ankara (MVA) virus from Bavarian Nordic A/S (Hellerup, Denmark) as a prime-boost immunization approach against COVID-19. The adenovirus type 26 vector was demonstrated to mediate protection against SIV in NHPs (76) and immunogenicity against Ebola virus in clinical phase I testing (133). Vaxart (South San Francisco, CA, USA) initiated a project to develop a COVID-19 vaccine based on the VAAST™ platform, which contains an adenovirus 5 vector and a TLR3 adjuvant, and it is designed as enteric-coated vaccine tablets that release the vector in the small intestine for targeted immune activation, as previously shown for an oral influenza candidate vaccine (140, 141). Imophoron's (Bristol, UK) in collaboration with University of Bristol (Bristol, UK) is using the ADDomer vaccine platform, which is an adenovirus-derived multimeric protein-based self-assembling nanoparticle scaffold engineered to facilitate plug-and-play display of multiple immunogenic epitopes from pathogens, and it has been tested against Chikungunya infection (142). ReiThera (Rome, Italy), LEUKOCARE (Munich, Germany), and Univercells (Brussels, Belgium) are developing a vaccine candidate based on ReiThera's simian adenoviral vector with strong immunological potency (143, 144) and Univercells's NevoLine™ biomanufacturing platform for scale up. GeoVax's (Atlanta, GE, USA) MVA platform technology has the advantage of being a live replication-competent vector in avian cells for manufacturing, yet replication-deficient in mammalian cells upon vaccination, and it was found to protect against Lassa fever virus in mice (134) and Ebola virus in NHPs (135). The DZIF is also developing a COVID-19 vaccine candidate based on MVA as a viral vector for the SARS-CoV-2 S protein, and protective efficacy against MERS infection has previously been demonstrated in mice (136) and camels (137). Medicago (Uppsala, Sweden) is using SARS-CoV-2 protein VLPs produced in tobacco (*Nicotiana Benthamiana*) to generate cellular and humoral immunity, as shown previously against influenza in clinical testing (138, 139). University of Georgia (Athens, GA, USA) in collaboration with University of Iowa (Iowa city, IA, USA) is developing a vaccine candidate using a parainfluenza virus 5 vector that encodes the S protein of SARS-CoV-2. Using this vector, a similar vaccine has been developed against MERS-CoV, which was protective in mice (145).

LV-SMENP-DC and pathogen-specific artificial antigen-presenting cell (aAPC) are the two lentiviral vector-based vaccine candidates in clinical trials from Shenzhen Geno-Immune Medical Institute (Guangdong, China) (**Table 2**). For the LV-SMENP-DC vaccine, an efficient lentiviral vector system (NHP/TYF) is used to express SARS-CoV-2 minigenes, engineered based on multiple viral genes, into viral proteins and immune-modulatory genes to modify DCs and to activate T cells (188, 189). In a similar strategy, a lentiviral vector system is used to express viral proteins and immune modulatory proteins to modify aAPC and to activate T cells (190). ChAdOx1 nCoV-19, developed by University of Oxford (Oxford, UK) and manufactured by Advent Srl (Pomezia, Italy), consists of an attenuated chimpanzee adenovirus capable of producing the S protein of SARS-CoV-2, and it is expected to induce antibodies against these proteins in SARS-CoV-2. The ChAdOx1 viral vector was shown to elicit nAbs and cellular immune responses in mice against human MERS-CoV (191). Another non-replicating viral vector-based vaccine candidate in clinical trials has been developed by CanSino Biologics (Hubei, China) and is based on a recombinant adenovirus type 5 vector (192).

Replicating Viral Vector Vaccines

Measles virus, influenza virus, vesicular stomatitis virus, and horse pox virus, respectively, are used as replicating viral vector platforms to develop novel COVID-19 vaccine candidates (**Table 1**). Institut Pasteur (Paris, France) is exploiting their measles vaccine vector technology and has developed vaccine candidates against chikungunya (146) and MERS (147) based on this technology. Tonix Pharmaceuticals (New York, NY, USA) in collaboration with Southern Research (Birmingham, Alabama, USA) is developing TNX-1800, which is a live modified horsepox virus designed to express the S protein of SARS-CoV-2, and it is based on Tonix's biodefense vaccines against small pox and monkey pox (148, 149). The International AIDS Vaccine Initiative (IAVI, New York, NY, USA) is exploiting a recombinant vesicular stomatitis virus (rVSV) vector against COVID-19 and has demonstrated efficacy of rVSV-vectored vaccines against SIV in NHPs (150) and Ebola virus in humans (151). CEPI is partnering with The University of Hong Kong (Hong Kong, China) to develop a COVID-19 vaccine candidate based on a live-attenuated influenza vaccine platform (152, 153). The University of Wisconsin–Madison (Madison, WI, USA) and the vaccine companies FluGen (Madison, WI, USA) and Bharat Biotech (Hyderabad, India) have initiated the development and testing of the vaccine candidate CoroFlu that builds on the backbone of FluGen's flu vaccine candidate known as M2SR, which is a self-limiting version of the influenza virus in which gene sequences of SARS-CoV-2 are inserted to induce additional immunity against coronavirus (154, 155). Although several viral vector-based COVID-19 vaccine candidates are in preclinical as well as clinical development, several drawbacks are associated with the use of viral vectors to deliver genetic material to cells. First, the viral vector itself can induce an immune response in the body (193). Second, if a vaccine fails during clinical testing, the same viral vector cannot be reused in the patient because it can induce an immune response. Third, pre-existing immunity against the

viral vector can render a vaccine ineffective (193). However, pre-existing immunity can be challenged by priming with a non-viral DNA vaccine (194) or by increasing the vaccine dose or changing the administration route (195). Other potential issues with viral vectors, e.g., low transgenic expression and genetic toxicity, can be overcome by using hybrid viral vectors (196).

DNA Vaccines

This type of vaccine contains selected gene(s) of the virus in the form of DNA. Upon injection, the DNA is used as template for *in situ* expression of potentially harmless viral protein(s), which induces a protective immune response. One of the greatest advantage of this type of vaccine is the safety and scalability for mass production. DNA-based viral vaccines have been shown to induce strong immune responses in animal models, especially in mice (100, 197–199). However, there is limited positive clinical data on DNA-based viral vaccines in humans, and no commercial DNA vaccine against any disease has yet been approved. Nevertheless, several DNA vaccine candidates are tested preclinically (**Figure 3A**) and two candidates have progressed into phase I clinical testing (**Figure 3B**). Zydus Cadila (Ahmedabad, India) is developing a DNA vaccine against the S protein of SARS-CoV-2 based on an indigenously developed plasmid DNA delivery technology (19). Evvivax (Rome, Italy) collaborates with Applied DNA Sciences (Stony Brook, NY, USA) and Takis Biotech (Rome, Italy) to develop four linear DNA-based vaccine candidates. Evvivax utilizes viral or plasmid DNA vectors for *in vivo* delivery of an expression cassette carrying the coding region of the target gene in combination with the electro-gene-transfer technology from Takis Biotech (heterologous prime/boost) (156, 157). AnGes Inc. (Osaka, Japan) in partnership with Osaka University is developing a DNA-based COVID-19 vaccine candidate based on a hepatocyte growth factor plasmid, which has been used to develop a therapeutic DNA vaccine against hypertension (158, 159). Cobra Biologics (Newcastle, UK) and Karolinska Institutet (Stockholm, Sweden) are developing a DNA vaccine candidate, which is based on Cobra's ORT® (Operator-Repressor Titration) technology for producing plasmid DNA without antibiotics, antibiotic resistance genes or any other selectable marker genes (160, 161). These vaccine strategies all involve DNA administration by conventional intramuscular immunization. However, Immunomic Therapeutics (Rockville, MD, USA) is working with EpiVax and PharmaJet (Golden, CO, USA) to develop a DNA vaccine that is delivered intradermally using a needle-free injection system. This partnership will combine platform technologies from all three companies: Immunomic's (Rockville, MD, USA) UNiversal Intracellular Targeted Expression (UNITE) platform (162), EpiVax's *in silico* T-cell epitope prediction tool (102, 103), and PharmaJet's Tropis® needle-free injection system that accurately targets delivery to the intradermal tissue layer (163). Immunomic's UNITE platform involves fusing pathogenic antigens with lysosomal-associated membrane protein, which is an endogenous protein in humans, for enhanced MHC-II processing and MHC-I cross presentation and subsequent induction of both Th1 and $CD8^+$ T cell responses (162). Researchers at the University of Waterloo (Waterloo, ON, Canada) are developing a DNA-based vaccine that is administered using a nasal spray. They will use a lambda bacteriophage system for delivering DNA into target cells (164, 165), which them produce SARS-CoV-2 VLPs that stimulate an immune response.

Among DNA vaccine candidates in clinical testing, bacTRL-Spike developed by Symvivo Corporation (Burnaby, Canada) is based on the bacTRL platform technology, which is a genetically modified live cell probiotic bacteria-based gene delivery platform (200). Each oral dose of bacTRL-Spike contains live *Bifidobacterium longum*, which has been genetically engineered to deliver plasmids containing synthetic DNA encoding the S protein of SARS-CoV-2. INO-4800 developed by Inovio Pharmaceuticals (Pennsylvania, USA) involves intradermal plasmid delivery directly into cells using INOVIO's proprietary hand-held device called CELLECTRA® 2000 (201, 202). The principle of CELLECTRA® 2000 is to use a brief electrical pulse to reversibly open small pores in the cell membrane to allow plasmid entry (203).

RNA Vaccines

Similar to DNA vaccines, RNA vaccines contain selected genes of the virus in the form of mRNA, and following cytosolic delivery, these genes are translated into viral proteins. The mRNA-1273 from Moderna Therapeutics (Cambridge, MA, USA) is the first candidate vaccine that entered into Phase I clinical testing just 42 days after the sequencing of the full SARS-CoV-2 genome (*ClinicalTrials.gov identifier NCT04283461*) (**Table 2**). The mRNA-1273 has recently entered into phase II clinical testing (*ClinicalTrials.gov identifier NCT04405076*). mRNA-1273 is a novel LNP-encapsulated, mRNA-based vaccine that encodes the full-length, prefusion-stabilized S protein of SARS-CoV-2. This LNP-based technology platform has previously been shown to induce strong immune responses and protection against a number of different pathogens in preclinical studies (204, 205). In addition, the LNP technology was approved in 2018 for siRNA delivery as part of the product Patisiran (Onpattro, Alnylam Pharmaceuticals, Cambridge, MA, USA), which inhibits hepatocyte expression of transthyretin in patients with hereditary transthyretin-mediated amyloidosis (206). The mRNA-based vaccine candidate program BNT162 of BioNTech's (Mainz, Germany), which is developed jointly with Pfizer, is based on BioNTech's extensive experience with developing mRNA-based therapeutics, in particular against cancer, using customized mRNA molecules and intracellular delivery systems (207–209). BNT162 comprises of four vaccine candidates, each of which represent different mRNA formats and target antigens (S and RBD), and they are formulated using the LNP delivery system. Two candidates include nucleoside-modified mRNA (modRNA), one includes a uridine-containing mRNA (uRNA), and the fourth vaccine candidate is based on saRNA. CureVac (Tübingen, Germany) is exploiting the propriety RNAoptimizer® platform technology for developing a novel COVID-19 vaccine candidate (**Table 1**). BIOCAD (Saint-Petersburg, Russia) is designing an mRNA vaccine against SARS-COV-2 based on previous experience with mRNA-based cancer vaccines (19). The mRNA COVID-19 vaccine candidate co-developed by the Chinese

Center for Disease Control and Prevention (Beijing, China), Tongji University School of Medicine (Shanghai, China), and Stermirna Therapeutics (Shanghai, China) is based on Stermirna's mRNA synthesis and lipopolyplex nano-delivery platform (19). Fudan University (Fudan, China), in cooperation with Shanghai JiaoTong University (Shanghai, China), and RNACure Biopharma (Shanghai, China), is pursuing two different strategies to develop mRNA vaccines against COVID-19. The first strategy includes an mRNA encoding the RBD of the S protein to induce nAbs (19), while the second strategy includes an mRNA that instructs the host to produce VLPs (19). Imperial College London (London, UK) is developing an mRNA COVID-19 vaccine based on prior work with lipid nanoparticle (LNP)-encapsulated self-amplifying RNA, which has previously been shown to induce antibodies against the HIV-1 Env gp140 (166, 167). Arcturus Therapeutics (San Diego, CA, USA) in collaboration with Duke National University of Singapore (Singapore) is developing a vaccine candidate using its STARR™ (self-transcribing and replicating RNA) technology platform that combines self-replicating RNA with the nanoparticle delivery system LUNAR® into a single solution for *in situ* expression of SARS-CoV-2 proteins that induce an anti-viral immune response (168). eTheRNA Immunotherapies (Niel, Belgium) is developing a novel vaccine using the proprietary TriMix technology platform (169, 170). The TriMix platform comprises three different mRNAs encoding proteins (caTLR4, CD40L, and CD70) that stimulates dendritic cells (DCs) to activate strong $CD4^+$ and CD8 T^+ cell responses, and it was shown to induce immunogenic responses in preclinical (169) and clinical studies (170) of an mRNA-based melanoma vaccine. Sanofi Pasteur and Translate Bio (Lexington, MA, USA) are collaborating to develop a novel mRNA vaccine based on Translate Bio's proprietary mRNA therapeutic platform (MRT™). This platform includes the design of the desired mRNA sequences and then packaging them into delivery systems (171), and it has been shown to induce therapeutic antibodies against human epidermal growth factor receptor 2-positive tumors in humanized mice (172). Centro Nacional de Biotecnología (Madrid, Spain) is developing an mRNA COVID-19 vaccine candidate based on the highly attenuated poxvirus vector MVA expressing the S protein, which has previously been tested as a vector for vaccine candidates against Zika and Ebola viruses (173, 174). Daiichi Sankyo (Tokyo, Japan) is developing an mRNA vaccine encoding the S protein using their novel nucleic acid delivery technology based on LNPs, and the protective effects of the vaccine will be verified in animal models in partnership with University of Tokyo (Tokyo, Japan) (175).

Repurposed Vaccines

Bacillus Calmette–Guérin (BCG), which is a live attenuated vaccine that was developed against tuberculosis, has been reported to decrease the susceptibility to respiratory tract infections (210, 211) through reprogramming of innate immunity (212). Currently, there is no evidence that the BCG vaccine affords protection against COVID-19. However, several phase III and IV clinical trials are investigating if the BCG vaccine can help to boost the immune system and reduce the infection rate of SARS-CoV-2 (*ClinicalTrials.gov identifier NCT04327206, NCT04348370, NCT04350931, NCT04362124, NCT04369794,* and *EU Clinical Trials Register 2020-001591-15, 020-001678-31*) or reduce absenteeism among healthcare workers involved in COVID-19 patient care (*NCT04328441* and *NCT04373291*). One phase I trial in China is testing the effect of inhalation of inactivated *Mycobacterium vaccae* on protection against COVID-19 (*Chinese Clinical Trials Register ChiCTR2000030016*).

VACCINE MANUFACTURE

Vaccine development and manufacture of sufficient doses to induce herd immunity is one of the most challenging tasks within biopharmaceutical enterprises due to the complexity of the products. The most basic requirements for manufacturing vaccines in a way that is safe, effective yet consistent from batch to batch are difficult to implement. A number of variables dictate the outcome of vaccine production processes, including (i) the biological variability of the starting material, (ii) the pathogen, (iii) the environmental conditions during culture, (iv) the expertise of the manufacturing personnel, and (v) multiple steps during the purification process. In addition to these variables, the analytical methods used and the antigens produced during manufacturing often have high intrinsic variability. Scale up and safety of vaccine formulations are equally important for maintaining a successful production process. Therefore, improved technologies to streamline vaccine development and manufacturing are crucial. During the past decades, multiple novel platforms have been developed for producing vaccines at pandemic speed, including VLPs, viral vector-based vaccines, and nucleic acid-based vaccines. Each platform has its own advantages and challenges related to its ability to induce potent immune responses, manufacturing capacity, and safety for clinical use (**Table 3**). Therefore, it is unlikely that any single platform on its own will constitute a solution for the ongoing COVID-19 pandemic or a pandemic situation in the future (213).

CONCLUDING REMARKS

Currently, SARS-CoV-2 is spreading and posing a considerable economic and public health concern globally. It is urgent to design and develop safe and efficacious vaccines to prevent further spread of COVID-19 and establish vaccine-induced herd immunity. The development of transforming vaccine technology platforms over the past few decades has broadened the scope and shortened the time from pathogen identification to the deployment of vaccine candidates for clinical testing. The global COVID-19 vaccine pipeline is currently expanding on a daily basis, and radical rethinking of vaccine development and manufacturing processes may substantially improve our responses to the COVID-19 pandemic. The knowledge generated through vaccine development efforts for

TABLE 3 | Vaccine platform technologies used for developing vaccines against COVID-19.

Platform	Antigen type	Immune response	Advantages	Disadvantages	Response time in pandemics
Inactivated (egg-based)	Inactivated pathogen	Humoral Cellular	Over 70 years of experience Potent Simple formulation	Labor-intensive Difficult to manufacture in a short time Stringent quality control	Low
Live attenuated	Attenuated pathogen	Humoral Cellular	Potent Multivalent by nature Simple formulation No adjuvants required	Labor-intensive Difficult to manufacture in a short time Stringent quality control Risk for infection	Low
Subunit/Recombinant protein	Protein	Humoral	Non-infectious Less side effects	Labor-intensive New production process and stability assays for each new antigen Quality control Cold chain transfer and storage Need for adjuvants	Medium
Virus-like particles (VLPs)	Protein	Humoral	Non-infectious Potent	Stability Quality control Potential contaminants Assembly into stable particles Heterogeneity Cold chain transfer and storage	Medium
Viral vectors	Nucleic acid	Humoral Cellular	Potent No need for an adjuvant Antigens are expressed natively	Recombination of virus during production Contaminants from human- or animal-derived material Pre-existing immunity against the vector	High
DNA	Nucleic acid	Humoral Cellular	Room temperature storage Rapid large-scale production Options for multivalency Cell-free No contaminants Non-infectious	Weak immunogenicity in humans Risk of carcinogenesis due to potential genetic integration Difficult to scale up to g-kg scale Purity High concentration	High
mRNA	Nucleic acid	Humoral Cellular	Room temperature storage Ease of large-scale production Options for multivalency Cell free No contaminants Non-infectious No genome integration risk No anti-vector immunity	Scale up of mRNA synthesis Stability Stringent RNase-free environment Relatively higher cost Risk of adverse reaction Inflammation	High

closely related coronavirus strains, e.g., SARS and MERS, are used to direct the vaccine development efforts for COVID-19. Although inducing nAbs against the S proteins represents the main target for the majority of the vaccine candidates, the prospects of exploiting T and B cell responses for COVID-19 vaccination should also be considered, because these responses have been found to be persistent and protective in animal models. In addition, strategies worth further investigation include (i) vaccine potentiation with adjuvants, (ii) tailoring of S protein, (iii) targeting RBD and N proteins, (iv) mucosal immunization, and (v) the employment of unchartered vaccine platforms for reducing vaccine development time and costs, and/or for improving vaccine safety and efficacy. The lack of naturally acquired immunity against SARS-CoV-2 should not be considered a bottleneck in developing efficacious vaccines against COVID-19, because this was disproved for vaccination against now eradicated smallpox. Also, research efforts should be directed toward studying SARS-CoV-2 infection in appropriate animal models to analyze (i) viral replication, (ii) transmission, (iii) pathogenesis, and (iv) host immune responses, as well as (v) the effect of serious underlying medical conditions like hypertension and diabetes. To date, no mRNA vaccines have been licensed for human use. However, important lessons from the current advancement of the mRNA vaccine platform technology for COVID-19, which takes place at an unprecedented pace, may benefit the development of any target vaccine in the future, because the technology implies a significantly reduced overall time from target identification to regulatory approval and deployment of the vaccine. Collectively, the broad array of platform technologies

under investigation in the development and manufacture of novel vaccines against SARS-CoV-2 will hopefully result in one or a few safe and efficacious novel COVID-19 vaccines that can bring us closer to the goal of COVID-19 herd immunity.

AUTHOR CONTRIBUTIONS

LF, YZ, and AT wrote the review article. CF and AT critically revised all versions of the article. All authors contributed to the article and approved the submitted version.

FUNDING

We gratefully acknowledge the financial support from the Independent Research Fund, Denmark, Technology and Production Sciences (Grant No. 9041-00198B). The funder had no role in literature search, decision to publish, or preparation of the article.

REFERENCES

1. Zhou P, Yang XL, Wang XG, Hu B, Zhang L, Zhang W, et al. A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature.* (2020) 579:270–3. doi: 10.1038/s41586-020-2012-7
2. Munster VJ, Koopmans M, van Doremalen N, van Riel D, de Wit E. A novel coronavirus emerging in China - key questions for impact assessment. *N Engl J Med.* (2020) 382:692–4. doi: 10.1056/NEJMp2000929
3. WHO. *Coronavirus Disease 2019 (COVID-19): Situation Report, 155. 2020.* (2020). Available online at: https://www.who.int/docs/default-source/coronaviruse/situation-reports/20200623-covid-19-sitrep-155.pdf?sfvrsn=ca01ebe_2 (accessed June 23, 2020).
4. Hui DS, Azhar EI, Madani TA, Ntoumi F, Kock R, Dar O, et al. The continuing 2019-nCoV epidemic threat of novel coronaviruses to global health - The latest 2019 novel coronavirus outbreak in Wuhan, China. *Int J Infect Dis.* (2020) 91:264–6. doi: 10.1016/j.ijid.2020.01.009
5. Greenwood B. The contribution of vaccination to global health: past, present and future. *Philos Trans R Soc Lond B Biol Sci.* (2014) 369:20130433. doi: 10.1098/rstb.2013.0433
6. WHO. *Immunization Coverage*. (2019). Available online at: https://www.who.int/en/news-room/fact-sheets/detail/immunization-coverage (accessed June 23, 2020).
7. Mallory ML, Lindesmith LC, Baric RS. Vaccination-induced herd immunity: successes and challenges. *J Allergy Clin Immunol.* (2018) 142:64–6. doi: 10.1016/j.jaci.2018.05.007
8. Plans P, Torner N, Godoy P, Jane M. Lack of herd immunity against measles in individuals aged <35 years could explain re-emergence of measles in Catalonia (Spain). *Int J Infect Dis.* (2014) 18:81–3. doi: 10.1016/j.ijid.2013.09.015
9. Kwok KO, Lai F, Wei WI, Wong SYS, Tang JWT. Herd immunity - estimating the level required to halt the COVID-19 epidemics in affected countries. *J Infect.* (2020) 80:e32–33. doi: 10.1016/j.jinf.2020.03.027
10. Fung TS, Liu DX. Human coronavirus: host-pathogen interaction. *Annu Rev Microbiol.* (2019) 73:529–57. doi: 10.1146/annurev-micro-020518-115759
11. Perlman S, Netland J. Coronaviruses post-SARS: update on replication and pathogenesis. *Nat Rev Microbiol.* (2009) 7:439–50. doi: 10.1038/nrmicro2147
12. Shereen MA, Khan S, Kazmi A, Bashir N, Siddique R. COVID-19 infection: origin, transmission, and characteristics of human coronaviruses. *J Adv Res.* (2020) 24:91–8. doi: 10.1016/j.jare.2020.03.005
13. Fehr AR, Perlman S. Coronaviruses: an overview of their replication and pathogenesis. *Methods Mol Biol.* (2015) 1282:1–23. doi: 10.1007/978-1-4939-2438-7_1
14. Kopecky-Bromberg SA, Martinez-Sobrido L, Frieman M, Baric RA, Palese P. Severe acute respiratory syndrome coronavirus open reading frame (ORF) 3b, ORF 6, and nucleocapsid proteins function as interferon antagonists. *J Virol.* (2007) 81:548–57. doi: 10.1128/JVI.01782-06
15. Birra D, Benucci M, Landolfi L, Merchionda A, Loi G, Amato P, et al. COVID 19: a clue from innate immunity. *Immunol Res.* (2020) 10:1–8. doi: 10.1007/s12026-020-09137-5
16. Walls AC, Park YJ, Tortorici MA, Wall A, McGuire AT, Veesler D. Structure, function, and antigenicity of the SARS-CoV-2 spike glycoprotein. *Cell.* (2020) 181:281–92.e6. doi: 10.1016/j.cell.2020.02.058
17. Su S, Wong G, Shi W, Liu J, Lai ACK, Zhou J, et al. Epidemiology, genetic recombination, and pathogenesis of coronaviruses. *Trends Microbiol.* (2016) 24:490–502. doi: 10.1016/j.tim.2016.03.003
18. Jin Y, Yang H, Ji W, Wu W, Chen S, Zhang W, et al. Virology, epidemiology, pathogenesis, and control of COVID-19. *Viruses.* (2020) 12:372. doi: 10.3390/v12040372
19. WHO. *DRAFT Landscape of COVID-19 Candidate Vaccines*. (2020) Available online at: https://www.who.int/publications/m/item/draft-landscape-of-covid-19-candidate-vaccines (accessed July 13, 2020).
20. Zhang J, Zeng H, Gu J, Li H, Zheng L, Zou Q. Progress and prospects on vaccine development against SARS-CoV-2. *Vaccines (Basel).* (2020) 8:E153. doi: 10.3390/vaccines8020153
21. Kim SJ, Nguyen VG, Park YH, Park BK, Chung HC. A novel synonymous mutation of SARS-CoV-2: is this possible to affect their antigenicity and immunogenicity? *Vaccines.* (2020) 8:E220. doi: 10.3390/vaccines8020220
22. Zhang T, Wu Q, Zhang Z. Probable pangolin origin of SARS-CoV-2 associated with the COVID-19 outbreak. *Curr Biol.* (2020) 30:1346–51.e2. doi: 10.1016/j.cub.2020.03.022
23. Andersen KG, Rambaut A, Lipkin WI, Holmes EC, Garry RF. The proximal origin of SARS-CoV-2. *Nat Med.* (2020) 26:450–2. doi: 10.1038/s41591-020-0820-9
24. Bedford J, Enria D, Giesecke J, Heymann DL, Ihekweazu C, Kobinger G, et al. COVID-19: towards controlling of a pandemic. *Lancet.* (2020) 395:1015–8. doi: 10.1016/S0140-6736(20)30673-5
25. van Doremalen N, Bushmaker T, Morris DH, Holbrook MG, Gamble A, Williamson BN, et al. Aerosol and surface stability of SARS-CoV-2 as compared with SARS-CoV-1. *N Engl J Med.* (2020) 382:1564–7. doi: 10.1056/NEJMc2004973
26. Wolfel R, Corman VM, Guggemos W, Seilmaier M, Zange S, Muller MA, et al. Virological assessment of hospitalized patients with COVID-2019. *Nature.* (2020) 581:465–469. doi: 10.1038/s41586-020-2196-x
27. Leung NHL, Chu DKW, Shiu EYC, Chan KH, McDevitt JJ, Hau BJP, et al. Respiratory virus shedding in exhaled breath and efficacy of face masks. *Nat Med.* (2020) 26:676–80. doi: 10.21203/rs.3.rs-16836/v1
28. Wu Y, Guo C, Tang L, Hong Z, Zhou J, Dong X, et al. Prolonged presence of SARS-CoV-2 viral RNA in faecal samples. *Lancet Gastroenterol Hepatol.* (2020) 5:434–5. doi: 10.1016/S2468-1253(20)30083-2
29. Xu Y, Li X, Zhu B, Liang H, Fang C, Gong Y, et al. Characteristics of pediatric SARS-CoV-2 infection and potential evidence for persistent fecal viral shedding. *Nat Med.* (2020) 26:502–5. doi: 10.1038/s41591-020-0817-4
30. Rothan HA, Byrareddy SN. The epidemiology and pathogenesis of coronavirus disease (COVID-19) outbreak. *J Autoimmun.* (2020) 109:102433. doi: 10.1016/j.jaut.2020.102433
31. Lauer SA, Grantz KH, Bi Q, Jones FK, Zheng Q, Meredith HR, et al. The incubation period of coronavirus disease 2019 (COVID-19) from publicly reported confirmed cases: estimation and application. *Ann Intern Med.* (2020) 10:M20-0504. doi: 10.7326/M20-0504
32. Wang W, Tang J, Wei F. Updated understanding of the outbreak of 2019 novel coronavirus (2019-nCoV) in Wuhan, China. *J Med Virol.* (2020) 92:441–7. doi: 10.1002/jmv.25689
33. Lu R, Zhao X, Li J, Niu P, Yang B, Wu H, et al. Genomic characterisation and epidemiology of 2019 novel coronavirus:

implications for virus origins and receptor binding. *Lancet.* (2020) 395:565–74. doi: 10.1016/S0140-6736(20)30251-8
34. Lu X, Zhang L, Du H, Zhang J, Li YY, Qu J, et al. SARS-CoV-2 infection in children. *N Engl J Med.* (2020) 382:1663–5. doi: 10.1056/NEJMc2005073
35. Lagunas-Rangel FA. Neutrophil-to-lymphocyte ratio and lymphocyte-to-C-reactive protein ratio in patients with severe coronavirus disease 2019 (COVID-19): a meta-analysis. *J Med Virol.* (2020). doi: 10.1002/jmv.25819. [Epub ahead of print].
36. Zheng M, Gao Y, Wang G, Song G, Liu S, Sun D, et al. Functional exhaustion of antiviral lymphocytes in COVID-19 patients. *Cell Mol Immunol.* (2020) 17:533–35. doi: 10.1038/s41423-020-0402-2
37. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet.* (2020) 395:497–506. doi: 10.1016/S0140-6736(20)30183-5
38. Moore BJB, June CH. Cytokine release syndrome in severe COVID-19. *Science.* (2020) 368:473–74. doi: 10.1126/science.abb8925
39. de Wit E, van Doremalen N, Falzarano D, Munster VJ. SARS and MERS: recent insights into emerging coronaviruses. *Nat Rev Microbiol.* (2016) 14:523–34. doi: 10.1038/nrmicro.2016.81
40. Mantlo E, Bukreyeva N, Maruyama J, Paessler S, Huang C. Antiviral activities of type I interferons to SARS-CoV-2 infection. *Antiviral Res.* (2020) 179:104811. doi: 10.1016/j.antiviral.2020.104811
41. Acharya D, Liu G, Gack MU. Dysregulation of type I interferon responses in COVID-19. *Nat Rev Immunol.* (2020) 20:397–8. doi: 10.1038/s41577-020-0346-x
42. Nikolich-Zugich J, Knox KS, Rios CT, Natt B, Bhattacharya D, Fain MJ. SARS-CoV-2 and COVID-19 in older adults: what we may expect regarding pathogenesis, immune responses, and outcomes. *Geroscience.* (2020) 42:505–14. doi: 10.1007/s11357-020-00186-0
43. Zust R, Cervantes-Barragan L, Habjan M, Maier R, Neuman BW, Ziebuhr J, et al. Ribose 2'-O-methylation provides a molecular signature for the distinction of self and non-self mRNA dependent on the RNA sensor Mda5. *Nat Immunol.* (2011) 12:137–43. doi: 10.1038/ni.1979
44. Sun L, Xing Y, Chen X, Zheng Y, Yang Y, Nichols DB, et al. Coronavirus papain-like proteases negatively regulate antiviral innate immune response through disruption of STING-mediated signaling. *PLoS ONE.* (2012) 7:e30802. doi: 10.1371/journal.pone.0030802
45. Jiang S, Hillyer C, Du L. Neutralizing antibodies against SARS-CoV-2 and other human coronaviruses. *Trends Immunol.* (2020) 14:355–9. doi: 10.1016/j.it.2020.04.008
46. Jiang S, Du L, Shi Z. An emerging coronavirus causing pneumonia outbreak in Wuhan, China: calling for developing therapeutic and prophylactic strategies. *Emerg Microbes Infect.* (2020) 9:275–7. doi: 10.1080/22221751.2020.1723441
47. Hoffmann M, Kleine-Weber H, Schroeder S, Kruger N, Herrler T, Erichsen S, et al. SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor. *Cell.* (2020) 181:271–80.e8. doi: 10.1016/j.cell.2020.02.052
48. Janice Oh HL, Ken-En Gan S, Bertoletti A, Tan YJ. Understanding the T cell immune response in SARS coronavirus infection. *Emerg Microbes Infect.* (2012) 1:e23. doi: 10.1038/emi.2012.26
49. Zheng HY, Zhang M, Yang CX, Zhang N, Wang XC, Yang XP, et al. Elevated exhaustion levels and reduced functional diversity of T cells in peripheral blood may predict severe progression in COVID-19 patients. *Cell Mol Immunol.* (2020) 17:541–3. doi: 10.1038/s41423-020-0401-3
50. Ouyang Y, Yin J, Wang W, Shi H, Shi Y, Xu B, et al. Down-regulated gene expression spectrum and immune responses changed during the disease progression in COVID-19 patients. *Clin Infect Dis.* (2020). doi: 10.1093/cid/ciaa462. [Epub ahead of print].
51. Li CK, Wu H, Yan H, Ma S, Wang L, Zhang M, et al. T cell responses to whole SARS coronavirus in humans. *J Immunol.* (2008) 181:5490–500. doi: 10.4049/jimmunol.181.8.5490
52. Grifoni A, Weiskopf D, Ramirez SI, Mateus J, Dan JM, Moderbacher CR, et al. Targets of T cell responses to SARS-CoV-2 coronavirus in humans with COVID-19 disease and unexposed individuals. *Cell.* (2020) 181:1489–1501.e15. doi: 10.1016/j.cell.2020.05.015
53. Belzung C, Lemoine M. Criteria of validity for animal models of psychiatric disorders: focus on anxiety disorders and depression. *Biol Mood Anxiety Disord.* (2011) 1:9. doi: 10.1186/2045-5380-1-9
54. Safronetz D, Geisbert TW, Feldmann H. Animal models for highly pathogenic emerging viruses. *Curr Opin Virol.* (2013) 3:205–9. doi: 10.1016/j.coviro.2013.01.001
55. McCray PB Jr, Pewe L, Wohlford-Lenane C, Hickey M, Manzel L, et al. Lethal infection of K18-hACE2 mice infected with severe acute respiratory syndrome coronavirus. *J Virol.* (2007) 81:813–21. doi: 10.1128/JVI.02012-06
56. Bao L, Deng W, Huang B, Gao H, Liu J, Ren L, et al. The pathogenicity of SARS-CoV-2 in hACE2 transgenic mice. *Nature.* (2020). doi: 10.1038/s41586-020-2312-y. [Epub ahead of print].
57. Imai Y, Kuba K, Rao S, Huan Y, Guo F, Guan B, et al. Angiotensin-converting enzyme 2 protects from severe acute lung failure. *Nature.* (2005) 436:112–6. doi: 10.1038/nature03712
58. Graham RL, Becker MM, Eckerle LD, Bolles M, Denison MR, Baric RS. A live, impaired-fidelity coronavirus vaccine protects in an aged, immunocompromised mouse model of lethal disease. *Nat Med.* (2012) 18:1820–6. doi: 10.1038/nm.2972
59. Roberts A, Deming D, Paddock CD, Cheng A, Yount B, Vogel L, et al. A mouse-adapted SARS-coronavirus causes disease and mortality in BALB/c mice. *PLoS Pathog.* (2007) 3:e5. doi: 10.1371/journal.ppat.0030005
60. Day CW, Baric R, Cai SX, Frieman M, Kumaki Y, Morrey JD, et al. A new mouse-adapted strain of SARS-CoV as a lethal model for evaluating antiviral agents *in vitro* and *in vivo*. *Virology.* (2009) 395:210–22. doi: 10.1016/j.virol.2009.09.023
61. Subbarao K, Roberts A. Is there an ideal animal model for SARS? *Trends Microbiol.* (2006) 14:299–303. doi: 10.1016/j.tim.2006.05.007
62. Roberts A, Paddock C, Vogel L, Butler E, Zaki S, Subbarao K. Aged BALB/c mice as a model for increased severity of severe acute respiratory syndrome in elderly humans. *J Virol.* (2005) 79:5833–8. doi: 10.1128/JVI.79.9.5833-5838.2005
63. Roberts A, Lamirande EW, Vogel L, Jackson JP, Paddock CD, Guarner J, et al. Animal models and vaccines for SARS-CoV infection. *Virus Res.* (2008) 133:20–32. doi: 10.1016/j.virusres.2007.03.025
64. Glass WG, Subbarao K, Murphy B, Murphy PM. Mechanisms of host defense following severe acute respiratory syndrome-coronavirus (SARS-CoV) pulmonary infection of mice. *J Immunol.* (2004) 173:4030–9. doi: 10.4049/jimmunol.173.6.4030
65. Cockrell AS, Yount BL, Scobey T, Jensen K, Douglas M, Beall A, et al. A mouse model for MERS coronavirus-induced acute respiratory distress syndrome. *Nat Microbiol.* (2016) 2:16226. doi: 10.1038/nmicrobiol.2016.226
66. Zhang Q, Shi J, Deng G, Guo J, Zeng X, He X, et al. H7N9 influenza viruses are transmissible in ferrets by respiratory droplet. *Science.* (2013) 341:410–4. doi: 10.1126/science.1240532
67. Imai M, Watanabe T, Hatta M, Das SC, Ozawa M, Shinya K, et al. Experimental adaptation of an influenza H5 HA confers respiratory droplet transmission to a reassortant H5 HA/H1N1 virus in ferrets. *Nature.* (2012) 486:420–8. doi: 10.1038/nature10831
68. Chu YK, Ali GD, Jia F, Li Q, Kelvin D, Couch RC, et al. The SARS-CoV ferret model in an infection-challenge study. *Virology.* (2008) 374:151–63. doi: 10.1016/j.virol.2007.12.032
69. Shi J, Wen Z, Zhong G, Yang H, Wang C, Huang B, et al. Susceptibility of ferrets, cats, dogs, and other domesticated animals to SARS-coronavirus 2. *Science.* (2020) 368:1016–20. doi: 10.1126/science.abb7015
70. Roberts A, Vogel L, Guarner J, Hayes N, Murphy B, Zaki S, et al. Severe acute respiratory syndrome coronavirus infection of golden Syrian hamsters. *J Virol.* (2005) 79:503–11. doi: 10.1128/JVI.79.1.503-511.2005
71. de Wit E, Prescott J, Baseler L, Bushmaker T, Thomas T, Lackemeyer MG, et al. The Middle East respiratory syndrome coronavirus (MERS-CoV) does not replicate in Syrian hamsters. *PLoS ONE.* (2013) 8:e69127. doi: 10.1371/journal.pone.0069127
72. Chan JF, Zhang AJ, Yuan S, Poon VK, Chan CC, Lee AC, et al. Simulation of the clinical and pathological manifestations of coronavirus disease 2019 (COVID-19) in golden Syrian hamster model: implications for disease pathogenesis and transmissibility. *Clin Infect Dis.* (2020). doi: 10.1093/cid/ciaa325. [Epub ahead of print].

73. Rockx B, Kuiken T, Herfst S, Bestebroer T, Lamers MM, Oude Munnink BB, et al. Comparative pathogenesis of COVID-19, MERS, and SARS in a nonhuman primate model. *Science.* (2020) 368:1012–15. doi: 10.1101/2020.03.17.995639
74. Ota M. Will we see protection or reinfection in COVID-19? *Nat Rev Immunol.* (2020) 17:1. doi: 10.1038/s41577-020-0316-3
75. Yu P, Qi F, Xu Y, Li F, Liu P, Liu J, et al. Age-related rhesus macaque models of COVID-19. *Animal Model Exp Med.* (2020) 3:93–7. doi: 10.1002/ame2.12108
76. Barouch DH, Alter G, Broge T, Linde C, Ackerman ME, Brown EP, et al. Protective efficacy of adenovirus/protein vaccines against SIV challenges in rhesus monkeys. *Science.* (2015) 349:320–4. doi: 10.1126/science.aab3886
77. Rappuoli R, Santoni A, Mantovani A. Vaccines: an achievement of civilization, a human right, our health insurance for the future. *J Exp Med.* (2018) 216:7–9. doi: 10.1084/jem.20182160
78. Delany I, Rappuoli R, De Gregorio E. Vaccines for the 21st century. *EMBO Mol Med.* (2014) 6:708–20. doi: 10.1002/emmm.201403876
79. Posadas-Herrera G, Inoue S, Fuke I, Muraki Y, Mapua CA, Khan AH, et al. Development and evaluation of a formalin-inactivated West Nile virus vaccine (WN-VAX) for a human vaccine candidate. *Vaccine.* (2010) 28:7939–46. doi: 10.1016/j.vaccine.2010.09.076
80. Muraki Y, Fujita T, Matsuura M, Fuke I, Manabe S, Ishikawa T, et al. The efficacy of inactivated west Nile vaccine (WN-VAX) in mice and monkeys. *Virol J.* (2015) 12:54. doi: 10.1186/s12985-015-0282-8
81. Vanlandingham DL, Keil SD, Horne KM, Pyles R, Goodrich RP, Higgs S. Photochemical inactivation of chikungunya virus in plasma and platelets using the Mirasol pathogen reduction technology system. *Transfusion.* (2013) 53:284–90. doi: 10.1111/j.1537-2995.2012.03717.x
82. Faddy HM, Prow NA, Fryk JJ, Hall RA, Keil SD, Goodrich RP, et al. The effect of riboflavin and ultraviolet light on the infectivity of arboviruses. *Transfusion.* (2015) 55:824–31. doi: 10.1111/trf.12899
83. Keil SD, Bowen R, Marschner S. Inactivation of Middle East respiratory syndrome coronavirus (MERS-CoV) in plasma products using a riboflavin-based and ultraviolet light-based photochemical treatment. *Transfusion.* (2016) 56:2948–52. doi: 10.1111/trf.13860
84. Hyer R, McGuire DK, Xing B, Jackson S, Janssen R. Safety of a two-dose investigational hepatitis B vaccine, HBsAg-1018, using a toll-like receptor 9 agonist adjuvant in adults. *Vaccine.* (2018) 36:2604–11. doi: 10.1016/j.vaccine.2018.03.067
85. Hyer RN, Janssen RS. Immunogenicity and safety of a 2-dose hepatitis B vaccine, HBsAg/CpG 1018, in persons with diabetes mellitus aged 60-70years. *Vaccine.* (2019) 37:5854–61. doi: 10.1016/j.vaccine.2019.08.005
86. Gao Q, Bao L, Mao H, Wang L, Xu K, Yang M, et al. Rapid development of an inactivated vaccine candidate for SARS-CoV-2. *Science.* (2020) 6:eabc1932. doi: 10.1126/science.abc1932
87. Stauft CB, Yang C, Coleman JR, Boltz D, Chin C, Kushnir A, et al. Live-attenuated H1N1 influenza vaccine candidate displays potent efficacy in mice and ferrets. *PLoS ONE.* (2019) 14:e0223784. doi: 10.1371/journal.pone.0223784
88. Mueller S, Stauft CB, Kalkeri R, Koidei F, Kushnir A, Tasker S, et al. A codon-pair deoptimized live-attenuated vaccine against respiratory syncytial virus is immunogenic and efficacious in non-human primates. *Vaccine.* (2020) 38:2943–8. doi: 10.1016/j.vaccine.2020.02.056
89. Malczyk AH, Kupke A, Prufer S, Scheuplein VA, Hutzler S, Kreuz D, et al. A highly immunogenic and protective Middle East respiratory syndrome coronavirus vaccine based on a recombinant measles virus vaccine platform. *J Virol.* (2015) 89:11654–67. doi: 10.1128/JVI.01815-15
90. Nurnberger C, Bodmer BS, Fiedler AH, Gabriel G, Muhlebach MD. A measles virus-based vaccine candidate mediates protection against zika virus in an allogeneic mouse pregnancy model. *J Virol.* (2019) 93:e01485–18. doi: 10.1128/JVI.01485-18
91. Gupta G, Giannino V, Rishi N, Glueck R. Immunogenicity of next-generation HPV vaccines in non-human primates: measles-vectored HPV vaccine versus Pichia pastoris recombinant protein vaccine. *Vaccine.* (2016) 34:4724–31. doi: 10.1016/j.vaccine.2016.07.051
92. Palomares LA, Srivastava IK, Ramirez OT, Cox MMJ. Glycobiotechnology of the insect cell-baculovirus expression system technology. *Adv Biochem Eng Biotechnol.* (2018). doi: 10.1007/10_2018_61. [Epub ahead of print].
93. Dunkle LM, Izikson R, Patriarca PA, Goldenthal KL, Muse D, Cox MMJ. Randomized comparison of immunogenicity and safety of quadrivalent recombinant versus inactivated influenza vaccine in healthy adults 18-49 years of age. *J Infect Dis.* (2017) 216:1219–26. doi: 10.1093/infdis/jix478
94. Madan A, Ferguson M, Rheault P, Seiden D, Toma A, Friel D, et al. Immunogenicity and safety of an AS03-adjuvanted H7N1 vaccine in adults 65years of age and older: a phase II, observer-blind, randomized, controlled trial. *Vaccine.* (2017) 35:1865–72. doi: 10.1016/j.vaccine.2017.02.057
95. Lodaya RN, Kanitkar AP, Friedrich K, Henson D, Yamagata R, Nuti S, et al. Formulation design, optimization and *in vivo* evaluations of an alpha-tocopherol-containing self-emulsified adjuvant system using inactivated influenza vaccine. *J Control Release.* (2019) 316:12–21. doi: 10.1016/j.jconrel.2019.10.042
96. Hobson-Peters J, Harrison JJ, Watterson D, Hazlewood JE, Vet LJ, Newton ND, et al. A recombinant platform for flavivirus vaccines and diagnostics using chimeras of a new insect-specific virus. *Sci Transl Med.* (2019) 11:eaax7888. doi: 10.1126/scitranslmed.aax7888
97. Schwarz TF, Huang LM, Valencia A, Panzer F, Chiu CH, Decreux A, et al. A ten-year study of immunogenicity and safety of the AS04-HPV-16/18 vaccine in adolescent girls aged 10-14 years. *Hum Vaccin Immunother.* (2019) 15:1970–9. doi: 10.1080/21645515.2019.1625644
98. Qiao YL, Wu T, Li RC, Hu YM, Wei LH, Li CG, et al. Efficacy, safety, and immunogenicity of an *Escherichia coli*-produced bivalent human papillomavirus vaccine: an interim analysis of a randomized clinical trial. *J Natl Cancer Inst.* (2020) 112:145–53. doi: 10.1093/jnci/djz074
99. Zhang J, Zhang XF, Huang SJ, Wu T, Hu YM, Wang ZZ, et al. Long-term efficacy of a hepatitis E vaccine. *N Engl J Med.* (2015) 372:914–22. doi: 10.1056/NEJMoa1406011
100. Wang L, Shi W, Joyce MG, Modjarrad K, Zhang Y, Leung K, et al. Evaluation of candidate vaccine approaches for MERS-CoV. *Nat Commun.* (2015) 6:7712. doi: 10.1038/ncomms8712
101. Adney DR, Wang L, van Doremalen N, Shi W, Zhang Y, Kong WP, et al. Efficacy of an adjuvanted Middle East respiratory syndrome coronavirus spike protein vaccine in dromedary camels and alpacas. *Viruses.* (2019) 11:212. doi: 10.3390/v11030212
102. Moise L, De Groot A, Marcello A, Tassone R, Martin W, Cousens L. Building better biotherapeutics and vaccines by design: EpiVax, Inc., an immunology company. *R I Med J.* (2013) 96:19–21.
103. Moise L, Cousens L, Fueyo J, De Groot AS. Harnessing the power of genomics and immunoinformatics to produce improved vaccines. *Expert Opin Drug Discov.* (2011) 6:9–15. doi: 10.1517/17460441.2011.534454
104. Moise L, Gutierrez A, Kibria F, Martin R, Tassone R, Liu R, et al. iVAX: An integrated toolkit for the selection and optimization of antigens and the design of epitope-driven vaccines. *Hum Vaccin Immunother.* (2015) 11:2312–21. doi: 10.1080/21645515.2015.1061159
105. Sautto GA, Kirchenbaum GA, Abreu RB, Ecker JW, Pierce SR, Kleanthous H, et al. A computationally optimized broadly reactive antigen subtype-specific influenza vaccine strategy elicits unique potent broadly neutralizing antibodies against hemagglutinin. *J Immunol.* (2020) 204:375–85. doi: 10.4049/jimmunol.1900379
106. Muthumani K, Falzarano D, Reuschel EL, Tingey C, Flingai S, Villarreal DO, et al. A synthetic consensus anti-spike protein DNA vaccine induces protective immunity against Middle East respiratory syndrome coronavirus in nonhuman primates. *Sci Transl Med.* (2015) 7:301ra132. doi: 10.1126/scitranslmed.aac7462
107. Prescott J, Falzarano D, de Wit E, Hardcastle K, Feldmann F, Haddock E, et al. Pathogenicity and viral shedding of MERS-CoV in immunocompromised rhesus macaques. *Front Immunol.* (2018) 9:205. doi: 10.3389/fimmu.2018.00205
108. Ichinohe T, Ainai A, Ami Y, Nagata N, Iwata N, Kawaguchi A, et al. Intranasal administration of adjuvant-combined vaccine protects monkeys from challenge with the highly pathogenic influenza A H5N1 virus. *J Med Virol.* (2010) 82:1754–61. doi: 10.1002/jmv.21824
109. Asahi-Ozaki Y, Itamura S, Ichinohe T, Strong P, Tamura S, Takahashi H, et al. Intranasal administration of adjuvant-combined recombinant influenza virus HA vaccine protects mice from the lethal H5N1 virus infection. *Microbes Infect.* (2006) 8:2706–14. doi: 10.1016/j.micinf.2006.07.018
110. Kim E, Erdos G, Huang S, Kenniston TW, Balmert SC, Carey CD, et al. Microneedle array delivered recombinant coronavirus vaccines: Immunogenicity and rapid translational development. *EBioMedicine.* (2020) 55:102743. doi: 10.1016/j.ebiom.2020.102743

111. Strbo N, Vaccari M, Pahwa S, Kolber MA, Doster MN, Fisher E, et al. Cutting edge: novel vaccination modality provides significant protection against mucosal infection by highly pathogenic simian immunodeficiency virus. *J Immunol.* (2013) 190:2495–9. doi: 10.4049/jimmunol.1202655
112. Strbo N, Garcia-Soto A, Schreiber TH, Podack ER. Secreted heat shock protein gp96-Ig: next-generation vaccines for cancer and infectious diseases. *Immunol Res.* (2013) 57:311–25. doi: 10.1007/s12026-013-8468-x
113. Chen WH, Chag SM, Poongavanam MV, Biter AB, Ewere EA, Rezende W, et al. Optimization of the production process and characterization of the yeast-expressed SARS-CoV recombinant receptor-binding domain (RBD219-N1), a SARS vaccine candidate. *J Pharm Sci.* (2017) 106:1961–70. doi: 10.1016/j.xphs.2017.04.037
114. Chen WH, Du L, Chag SM, Ma C, Tricoche N, Tao X, et al. Yeast-expressed recombinant protein of the receptor-binding domain in SARS-CoV spike protein with deglycosylated forms as a SARS vaccine candidate. *Hum Vaccin Immunother.* (2014) 10:648–58. doi: 10.4161/hv.27464
115. Langley JM, MacDonald LD, Weir GM, MacKinnon-Cameron D, Ye L, McNeil S, et al. A respiratory syncytial virus vaccine based on the small hydrophobic protein ectodomain presented with a novel lipid-based formulation is highly immunogenic and safe in adults: a first-in-humans study. *J Infect Dis.* (2018) 218:378–87. doi: 10.1093/infdis/jiy177
116. Weir GM, MacDonald LD, Rajagopalan R, Sivko GS, Valderas MW, Rayner J, et al. Single dose of DPX-rPA, an enhanced-delivery anthrax vaccine formulation, protects against a lethal bacillus anthracis spore inhalation challenge. *NPJ Vaccines.* (2019) 4:6. doi: 10.1038/s41541-019-0102-z
117. Kovjazin R, Volovitz I, Kundel Y, Rosenbaum E, Medalia G, Horn G, et al. ImMucin: a novel therapeutic vaccine with promiscuous MHC binding for the treatment of MUC1-expressing tumors. *Vaccine.* (2011) 29:4676–86. doi: 10.1016/j.vaccine.2011.04.103
118. Carmon L, Avivi I, Kovjazin R, Zuckerman T, Dray L, Gatt ME, et al. Phase I/II study exploring ImMucin, a pan-major histocompatibility complex, anti-MUC1 signal peptide vaccine, in multiple myeloma patients. *Br J Haematol.* (2015) 169:44–56. doi: 10.1111/bjh.13245
119. Cunha-Neto E, Rosa DS, Harris PE, Olson T, Morrow A, Ciotlos S, et al. An approach for a synthetic CTL vaccine design against zika flavivirus using class I and class II epitopes identified by computer modeling. *Front Immunol.* (2017) 8:640. doi: 10.3389/fimmu.2017.00640
120. Rubsamen RM, Herst CV, Lloyd PM, Heckerman DE. Eliciting cytotoxic T-lymphocyte responses from synthetic vectors containing one or two epitopes in a C57BL/6 mouse model using peptide-containing biodegradable microspheres and adjuvants. *Vaccine.* (2014) 32:4111–6. doi: 10.1016/j.vaccine.2014.05.071
121. Xu M, Lu X, Sposato M, Zinckgraf JW, Wu S, von Hofe E. Ii-Key/HPV16 E7 hybrid peptide immunotherapy for HPV16+ cancers. *Vaccine.* (2009) 27:4641–7. doi: 10.1016/j.vaccine.2009.05.054
122. Perez SA, Kallinteris NL, Bisias S, Tzonis PK, Georgakopoulou K, Varla-Leftherioti M, et al. Results from a phase I clinical study of the novel Ii-Key/HER-2/neu(776-790) hybrid peptide vaccine in patients with prostate cancer. *Clin Cancer Res.* (2010) 16:3495–506. doi: 10.1158/1078-0432.CCR-10-0085
123. Garg R, Babiuk L, van Drunen Littel-van den Hurk S, Gerdts V. A novel combination adjuvant platform for human and animal vaccines. *Vaccine.* (2017) 35(35 Pt A):4486–9. doi: 10.1016/j.vaccine.2017.05.067
124. Garlapati S, Eng NF, Kiros TG, Kindrachuk J, Mutwiri GK, Hancock RE, et al. Immunization with PCEP microparticles containing pertussis toxoid, CpG ODN and a synthetic innate defense regulator peptide induces protective immunity against pertussis. *Vaccine.* (2011) 29:6540–8. doi: 10.1016/j.vaccine.2011.07.009
125. Kajikawa A, Zhang L, LaVoy A, Bumgardner S, Klaenhammer TR, Dean GA. Mucosal immunogenicity of genetically modified lactobacillus acidophilus expressing an HIV-1 epitope within the surface layer protein. *PLoS ONE.* (2015) 10:e0141713. doi: 10.1371/journal.pone.0141713
126. Bumgardner SA, Zhang L, LaVoy AS, Andre B, Frank CB, Kajikawa A, et al. Nod2 is required for antigen-specific humoral responses against antigens orally delivered using a recombinant lactobacillus vaccine platform. *PLoS ONE.* (2018) 13:e0196950. doi: 10.1371/journal.pone.0196950
127. Hjerrild KA, Jin J, Wright KE, Brown RE, Marshall JM, Labbe GM, et al. Production of full-length soluble Plasmodium falciparum RH5 protein vaccine using a drosophila melanogaster Schneider 2 stable cell line system. *Sci Rep.* (2016) 6:30357. doi: 10.1038/srep30357
128. Mordmuller B, Sulyok M, Egger-Adam D, Resende M, de Jongh WA, Jensen MH, et al. First-in-human, randomized, double-blind clinical trial of differentially adjuvanted PAMVAC, a vaccine candidate to prevent pregnancy-associated malaria. *Clin Infect Dis.* (2019) 69:1509–16. doi: 10.1093/cid/ciy1140
129. Escolano A, Gristick HB, Abernathy ME, Merkenschlager J, Gautam R, Oliveira TY, et al. Immunization expands B cells specific to HIV-1 V3 glycan in mice and macaques. *Nature.* (2019) 570:468–73. doi: 10.1038/s41586-019-1250-z
130. Janitzek CM, Peabody J, Thrane S, P HRC, T GT, Salanti A, et al. A proof-of-concept study for the design of a VLP-based combinatorial HPV and placental malaria vaccine. *Sci Rep.* (2019) 9:5260. doi: 10.1038/s41598-019-41522-5
131. Schwestka J, Tschofen M, Vogt S, Marcel S, Grillari J, Raith M, et al. Plant-derived protein bodies as delivery vehicles for recombinant proteins into mammalian cells. *Biotechnol Bioeng.* (2020) 117:1037–47. doi: 10.1002/bit.27273
132. Laughlin RC, Madera R, Peres Y, Berquist BR, Wang L, Buist S, et al. Plant-made E2 glycoprotein single-dose vaccine protects pigs against classical swine fever. *Plant Biotechnol J.* (2019) 17:410–20. doi: 10.1111/pbi.12986
133. Milligan ID, Gibani MM, Sewell R, Clutterbuck EA, Campbell D, Plested E, et al. Safety and immunogenicity of novel adenovirus type 26- and modified vaccinia Ankara-vectored Ebola vaccines: a randomized clinical trial. *JAMA.* (2016) 315:1610–23. doi: 10.1001/jama.2016.4218
134. Salvato MS, Domi A, Guzman-Cardozo C, Medina-Moreno S, Zapata JC, Hsu H, et al. A single dose of modified vaccinia Ankara expressing Lassa virus-like particles protects mice from lethal intra-cerebral virus challenge. *Pathogens.* (2019) 8:33. doi: 10.3390/pathogens8030133
135. Domi A, Feldmann F, Basu R, McCurley N, Shifflett K, Emanuel J, et al. A single dose of modified vaccinia Ankara expressing Ebola virus like particles protects nonhuman primates from lethal Ebola virus challenge. *Sci Rep.* (2018) 8:864. doi: 10.1038/s41598-017-19041-y
136. Volz A, Kupke A, Song F, Jany S, Fux R, Shams-Eldin H, et al. Protective efficacy of recombinant modified vaccinia virus Ankara delivering Middle East respiratory syndrome coronavirus spike glycoprotein. *J Virol.* (2015) 89:8651–6. doi: 10.1128/JVI.00614-15
137. Haagmans BL, van den Brand JM, Raj VS, Volz A, Wohlsein P, Smits SL, et al. An orthopoxvirus-based vaccine reduces virus excretion after MERS-CoV infection in dromedary camels. *Science.* (2016) 351:77–81. doi: 10.1126/science.aad1283
138. Landry N, Pillet S, Favre D, Poulin JF, Trepanier S, Yassine-Diab B, et al. Influenza virus-like particle vaccines made in nicotiana benthamiana elicit durable, poly-functional and cross-reactive T cell responses to influenza HA antigens. *Clin Immunol.* (2014) 154:164–77. doi: 10.1016/j.clim.2014.08.003
139. Pillet S, Couillard J, Trepanier S, Poulin JF, Yassine-Diab B, Guy B, et al. Immunogenicity and safety of a quadrivalent plant-derived virus like particle influenza vaccine candidate-two randomized Phase II clinical trials in 18 to 49 and >/=50 years old adults. *PLoS ONE.* (2019) 14:e0216533. doi: 10.1371/journal.pone.0216533
140. Scallan CD, Tingley DW, Lindbloom JD, Toomey JS, Tucker SN. An adenovirus-based vaccine with a double-stranded RNA adjuvant protects mice and ferrets against H5N1 avian influenza in oral delivery models. *Clin Vaccine Immunol.* (2013) 20:85–94. doi: 10.1128/CVI.00552-12
141. Liebowitz D, Gottlieb K, Kolhatkar NS, Garg SJ, Asher JM, Nazareno J, et al. Efficacy, immunogenicity, and safety of an oral influenza vaccine: a placebo-controlled and active-controlled phase 2 human challenge study. *Lancet Infect Dis.* (2020) 20:435–44. doi: 10.1016/S1473-3099(19)30584-5
142. Vragniau C, Bufton JC, Garzoni F, Stermann E, Rabi F, Terrat C, et al. Synthetic self-assembling ADDomer platform for highly efficient vaccination by genetically encoded multiepitope display. *Sci Adv.* (2019) 5:eaaw2853. doi: 10.1126/sciadv.aaw2853
143. Green CA, Sande CJ, Scarselli E, Capone S, Vitelli A, Nicosia A, et al. Novel genetically-modified chimpanzee adenovirus and MVA-vectored respiratory syncytial virus vaccine safely boosts humoral and

cellular immunity in healthy older adults. *J Infect.* (2019) 78:382–92. doi: 10.1016/j.jinf.2019.02.003

144. Stanley DA, Honko AN, Asiedu C, Trefry JC, Lau-Kilby AW, Johnson JC, et al. Chimpanzee adenovirus vaccine generates acute and durable protective immunity against ebolavirus challenge. *Nat Med.* (2014) 20:1126–9. doi: 10.1038/nm.3702
145. Li K, Li Z, Wohlford-Lenane C, Meyerholz DK, Channappanavar R, An D, et al. Single-dose, intranasal immunization with recombinant parainfluenza virus 5 expressing Middle East respiratory syndrome coronavirus (MERS-CoV) spike protein protects mice from fatal MERS-CoV infection. *mBio.* (2020) 11:e00554–20. doi: 10.1128/mBio.00554-20
146. Ramsauer K, Schwameis M, Firbas C, Mullner M, Putnak RJ, Thomas SJ, et al. Immunogenicity, safety, and tolerability of a recombinant measles-virus-based chikungunya vaccine: a randomised, double-blind, placebo-controlled, active-comparator, first-in-man trial. *Lancet Infect Dis.* (2015) 15:519–27. doi: 10.1016/S1473-3099(15)70043-5
147. Escriou N, Callendret B, Lorin V, Combredet C, Marianneau P, Fevrier M, et al. Protection from SARS coronavirus conferred by live measles vaccine expressing the spike glycoprotein. *Virology.* (2014) 452-453:32–41. doi: 10.1016/j.virol.2014.01.002
148. Noyce RS, Lederman S, Evans DH. Construction of an infectious horsepox virus vaccine from chemically synthesized DNA fragments. *PLoS ONE.* (2018) 13:e0188453. doi: 10.1371/journal.pone.0188453
149. Tulman ER, Delhon G, Afonso CL, Lu Z, Zsak L, Sandybaev NT, et al. Genome of horsepox virus. *J Virol.* (2006) 80:9244–58. doi: 10.1128/JVI.00945-06
150. Clarke DK, Nasar F, Chong S, Johnson JE, Coleman JW, Lee M, et al. Neurovirulence and immunogenicity of attenuated recombinant vesicular stomatitis viruses in nonhuman primates. *J Virol.* (2014) 88:6690–701. doi: 10.1128/JVI.03441-13
151. Henao-Restrepo AM, Camacho A, Longini IM, Watson CH, Edmunds WJ, Egger M, et al. Efficacy and effectiveness of an rVSV-vectored vaccine in preventing Ebola virus disease: final results from the guinea ring vaccination, open-label, cluster-randomised trial (Ebola Ca Suffit!). *Lancet.* (2017) 389:505–18. doi: 10.1016/S0140-6736(16)32621-6
152. Wang P, Zheng M, Lau SY, Chen P, Mok BW, Liu S, et al. Generation of DelNS1 influenza viruses: a strategy for optimizing live attenuated influenza vaccines. *mBio.* (2019) 10:e02180–19. doi: 10.1128/mBio.02180-19
153. Zheng M, Wang P, Song W, Lau SY, Liu S, Huang X, et al. An A14U substitution in the 3' noncoding region of the M segment of viral RNA supports replication of influenza virus with an NS1 deletion by modulating alternative splicing of M segment mRNAs. *J Virol.* (2015) 89:10273–85. doi: 10.1128/JVI.00919-15
154. Hatta Y, Boltz D, Sarawar S, Kawaoka Y, Neumann G, Bilsel P. M2SR, a novel live influenza vaccine, protects mice and ferrets against highly pathogenic avian influenza. *Vaccine.* (2017) 35:4177–83. doi: 10.1016/j.vaccine.2017.06.039
155. Hatta Y, Boltz D, Sarawar S, Kawaoka Y, Neumann G, Bilsel P. Novel influenza vaccine M2SR protects against drifted H1N1 and H3N2 influenza virus challenge in ferrets with pre-existing immunity. *Vaccine.* (2018) 36:5097–103. doi: 10.1016/j.vaccine.2018.06.053
156. Aurisicchio L, Peruzzi D, Koo G, Wei WZ, La Monica N, Ciliberto G. Immunogenicity and therapeutic efficacy of a dual-component genetic cancer vaccine cotargeting carcinoembryonic antigen and HER2/neu in preclinical models. *Hum Gene Ther.* (2014) 25:121–31. doi: 10.1089/hum.2013.103
157. Aurisicchio L, Fridman A, Bagchi A, Scarselli E, La Monica N, Ciliberto G. A novel minigene scaffold for therapeutic cancer vaccines. *Oncoimmunology.* (2014) 3:e27529. doi: 10.4161/onci.27529
158. Shigematsu H, Yasuda K, Sasajima T, Takano T, Miyata T, Ohta T, et al. Transfection of human HGF plasmid DNA improves limb salvage in buerger's disease patients with critical limb ischemia. *Int Angiol.* (2011) 30:140–9.
159. Koriyama H, Nakagami H, Nakagami F, Osako MK, Kyutoku M, Shimamura M, et al. Long-term reduction of high blood pressure by angiotensin II DNA vaccine in spontaneously hypertensive rats. *Hypertension.* (2015) 66:167–74. doi: 10.1161/HYPERTENSIONAHA.114.04534
160. Leckenby MW, Spear AM, Neeson BN, Williamson ED, Cranenburgh RM, Atkins HS. Enhanced vaccine antigen delivery by Salmonella using antibiotic-free operator-repressor titration-based plasmid stabilisation compared to chromosomal integration. *Microb Pathog.* (2009) 46:201–6. doi: 10.1016/j.micpath.2009.01.001
161. Huang JM, Sali M, Leckenby MW, Radford DS, Huynh HA, Delogu G, et al. Oral delivery of a DNA vaccine against tuberculosis using operator-repressor titration in a salmonella enterica vector. *Vaccine.* (2010) 28:7523–8. doi: 10.1016/j.vaccine.2010.09.001
162. Su Y, Connolly M, Marketon A, Heiland T. CryJ-LAMP DNA vaccines for Japanese red cedar allergy induce robust Th1-type immune responses in murine model. *J Immunol Res.* (2016) 2016:4857869. doi: 10.1155/2016/4857869
163. Resik S, Tejeda A, Mach O, Sein C, Molodecky N, Jarrahian C, et al. Needle-free jet injector intradermal delivery of fractional dose inactivated poliovirus vaccine: association between injection quality and immunogenicity. *Vaccine.* (2015) 33:5873–7. doi: 10.1016/j.vaccine.2015.06.071
164. Barati N, Razazan A, Nicastro J, Slavcev R, Arab A, Mosaffa F, et al. Immunogenicity and antitumor activity of the superlytic lambdaF7 phage nanoparticles displaying a HER2/neu-derived peptide AE37 in a tumor model of BALB/c mice. *Cancer Lett.* (2018) 424:109–16. doi: 10.1016/j.canlet.2018.03.030
165. Razazan A, Nicastro J, Slavcev R, Barati N, Arab A, Mosaffa F, et al. Lambda bacteriophage nanoparticles displaying GP2, a HER2/neu derived peptide, induce prophylactic and therapeutic activities against TUBO tumor model in mice. *Sci Rep.* (2019) 9:2221. doi: 10.1038/s41598-018-38371-z
166. Blakney AK, McKay PF, Yus BI, Aldon Y, Shattock RJ. Inside out: optimization of lipid nanoparticle formulations for exterior complexation and *in vivo* delivery of saRNA. *Gene Ther.* (2019) 26:363–72. doi: 10.1038/s41434-019-0095-2
167. Blakney AK, Yilmaz G, McKay PF, Becer CR, Shattock RJ. One size does not fit all: the effect of chain length and charge density of poly(ethylene imine) based copolymers on delivery of pDNA, mRNA, and RepRNA polyplexes. *Biomacromolecules.* (2018) 19:2870–9. doi: 10.1021/acs.biomac.8b00429
168. Ramaswamy S, Tonnu N, Tachikawa K, Limphong P, Vega JB, Karmali PP, et al. Systemic delivery of factor IX messenger RNA for protein replacement therapy. *Proc Natl Acad Sci U S A.* (2017) 114:E1941–50. doi: 10.1073/pnas.1619653114
169. Van Lint S, Goyvaerts C, Maenhout S, Goethals L, Disy A, Benteyn D, et al. Preclinical evaluation of TriMix and antigen mRNA-based antitumor therapy. *Cancer Res.* (2012) 72:1661–71. doi: 10.1158/0008-5472.CAN-11-2957
170. Wilgenhof S, Van Nuffel AM, Benteyn D, Corthals J, Aerts C, Heirman C, et al. A phase IB study on intravenous synthetic mRNA electroporated dendritic cell immunotherapy in pretreated advanced melanoma patients. *Ann Oncol.* (2013) 24:2686–93. doi: 10.1093/annonc/mdt245
171. Kauffman KJ, Dorkin JR, Yang JH, Heartlein MW, DeRosa F, Mir FF, et al. Optimization of lipid nanoparticle formulations for mRNA delivery *in vivo* with fractional factorial and definitive screening designs. *Nano Lett.* (2015) 15:7300–6. doi: 10.1021/acs.nanolett.5b02497
172. Rybakova Y, Kowalski PS, Huang Y, Gonzalez JT, Heartlein MW, DeRosa F, et al. mRNA delivery for therapeutic anti-HER2 antibody expression *in vivo*. *Mol Ther.* (2019) 27:1415–23. doi: 10.1016/j.ymthe.2019.05.012
173. Perez P, M QM, Lazaro-Frias A, Jimenez de Oya N, Blazquez AB, Escribano-Romero E, et al. A vaccine based on a modified vaccinia virus Ankara vector expressing zika virus structural proteins controls zika virus replication in mice. *Sci Rep.* (2018) 8:17385. doi: 10.1038/s41598-018-35724-6
174. Lazaro-Frias A, Gomez-Medina S, Sanchez-Sampedro L, Ljungberg K, Ustav M, Liljestrom P, et al. Distinct immunogenicity and efficacy of poxvirus-based vaccine candidates against Ebola virus expressing GP and VP40 proteins. *J Virol.* (2018) 92:e00363–18. doi: 10.1128/JVI.00363-18

175. Marzi A, Halfmann P, Hill-Batorski L, Feldmann F, Shupert WL, Neumann G, et al. Vaccines. An Ebola whole-virus vaccine is protective in nonhuman primates. *Science.* (2015) 348:439–42. doi: 10.1126/science.aaa4919
176. Shen Y, Hu Y, Meng F, Du W, Li W, Song Y, et al. Safety, immunogenicity and cross-reactivity of a Northern hemisphere 2013-2014 seasonal trivalent inactivated split influenza virus vaccine, Anflu(R). *Hum Vaccin Immunother.* (2016) 12:1229–34. doi: 10.1080/21645515.2015.1123357
177. Lin JT, Zhang JS, Su N, Xu JG, Wang N, Chen JT, et al. Safety and immunogenicity from a phase I trial of inactivated severe acute respiratory syndrome coronavirus vaccine. *Antivir Ther.* (2007) 12:1107–13.
178. Chen Y, Li L. SARS-CoV-2: virus dynamics and host response. *Lancet Infect Dis.* (2020) 20:515–16. doi: 10.1016/S1473-3099(20)30235-8
179. Xing YH, Ni W, Wu Q, Li WJ, Li GJ, Wang WD, et al. Prolonged viral shedding in feces of pediatric patients with coronavirus disease 2019. *J Microbiol Immunol Infect.* (2020) 53:473–80. doi: 10.1016/j.jmii.2020.03.021
180. Zhang N, Zheng BJ, Lu L, Zhou Y, Jiang S, Du L. Advancements in the development of subunit influenza vaccines. *Microbes Infect.* (2015) 17:123–34. doi: 10.1016/j.micinf.2014.12.006
181. Ou X, Liu Y, Lei X, Li P, Mi D, Ren L, et al. Characterization of spike glycoprotein of SARS-CoV-2 on virus entry and its immune cross-reactivity with SARS-CoV. *Nat Commun.* (2020) 11:1620. doi: 10.1038/s41467-020-15562-9
182. Liu H, Su D, Zhang J, Ge S, Li Y, Wang F, et al. Improvement of pharmacokinetic profile of TRAIL via Trimer-Tag enhances its antitumor activity *in vivo*. *Sci Rep.* (2017) 7:8953. doi: 10.1038/s41598-017-09518-1
183. Cui X, Chang L, Li Y, Lv Q, Wang F, Lin Y, et al. Trivalent soluble TNF receptor, a potent TNF-alpha antagonist for the treatment collagen-induced arthritis. *Sci Rep.* (2018) 8:7327. doi: 10.1038/s41598-018-25652-w
184. Chappell KJ, Watterson D, Young PR. *Inventors Chimeric Molecules and Uses Thereof.* U.S. Patent No. WO/2018/176103. St. Lucia, QLD: The University of Queensland (2018).
185. Garg R, Brownlie R, Latimer L, Gerdts V, Potter A, van Drunen Littel-van den Hurk S. A chimeric glycoprotein formulated with a combination adjuvant induces protective immunity against both human respiratory syncytial virus and parainfluenza virus type 3. *Antiviral Res.* (2018) 158:78–87. doi: 10.1016/j.antiviral.2018.07.021
186. Raghunandan R, Lu H, Zhou B, Xabier MG, Massare MJ, Flyer DC, et al. An insect cell derived respiratory syncytial virus (RSV) F nanoparticle vaccine induces antigenic site II antibodies and protects against RSV challenge in cotton rats by active and passive immunization. *Vaccine.* (2014) 32:6485–92. doi: 10.1016/j.vaccine.2014.09.030
187. Magnusson SE, Reimer JM, Karlsson KH, Lilja L, Bengtsson KL, Stertman L. Immune enhancing properties of the novel Matrix-M adjuvant leads to potentiated immune responses to an influenza vaccine in mice. *Vaccine.* (2013) 31:1725–33. doi: 10.1016/j.vaccine.2013.01.039
188. Chang LJ. Lentiviral vector transduction of dendritic cells for novel vaccine strategies. *Methods Mol Biol.* (2010) 614:161–71. doi: 10.1007/978-1-60761-533-0_11
189. Wang B, He J, Liu C, Chang LJ. An effective cancer vaccine modality: lentiviral modification of dendritic cells expressing multiple cancer-specific antigens. *Vaccine.* (2006) 24:3477–89. doi: 10.1016/j.vaccine.2006.02.025
190. Han S, Chang LJ. Immunity of lentiviral vector-modified dendritic cells. *Methods Mol Biol.* (2009) 542:245–59. doi: 10.1007/978-1-59745-561-9_13
191. Alharbi NK, Padron-Regalado E, Thompson CP, Kupke A, Wells D, Sloan MA, et al. ChAdOx1 and MVA based vaccine candidates against MERS-CoV elicit neutralising antibodies and cellular immune responses in mice. *Vaccine.* (2017) 35:3780–8. doi: 10.1016/j.vaccine.2017.05.032
192. Zhu FC, Wurie AH, Hou LH, Liang Q, Li YH, Russell JB, et al. Safety and immunogenicity of a recombinant adenovirus type-5 vector-based Ebola vaccine in healthy adults in Sierra Leone: a single-centre, randomised, double-blind, placebo-controlled, phase 2 trial. *Lancet.* (2017) 389:621–8. doi: 10.1016/S0140-6736(16)32617-4
193. Nayak S, Herzog RW. Progress and prospects: immune responses to viral vectors. *Gene Ther.* (2010) 17:295–304. doi: 10.1038/gt.2009.148
194. Yang ZY, Wyatt LS, Kong WP, Moodie Z, Moss B, Nabel GJ. Overcoming immunity to a viral vaccine by DNA priming before vector boosting. *J Virol.* (2003) 77:799–803. doi: 10.1128/JVI.77.1.799-803.2003
195. Pandey A, Singh N, Vemula SV, Couetil L, Katz JM, Donis R, et al. Impact of preexisting adenovirus vector immunity on immunogenicity and protection conferred with an adenovirus-based H5N1 influenza vaccine. *PLoS ONE.* (2012) 7:e33428. doi: 10.1371/journal.pone.0033428
196. Huang S, Kamihira M. Development of hybrid viral vectors for gene therapy. *Biotechnol Adv.* (2013) 31:208–23. doi: 10.1016/j.biotechadv.2012.10.001
197. Larocca RA, Abbink P, Peron JP, Zanotto PM, Iampietro MJ, Badamchi-Zadeh A, et al. Vaccine protection against zika virus from Brazil. *Nature.* (2016) 536:474–8. doi: 10.1038/nature18952
198. Chen MW, Cheng TJ, Huang Y, Jan JT, Ma SH, Yu AL, et al. A consensus-hemagglutinin-based DNA vaccine that protects mice against divergent H5N1 influenza viruses. *Proc Natl Acad Sci U S A.* (2008) 105:13538–43. doi: 10.1073/pnas.0806901105
199. Escolano A, Steichen JM, Dosenovic P, Kulp DW, Golijanin J, Sok D, et al. Sequential immunization elicits broadly neutralizing anti-HIV-1 antibodies in Ig knockin mice. *Cell.* (2016) 166:1445–58.e12. doi: 10.1016/j.cell.2016.07.030
200. rd Annual meeting & pre-conference programs of the society for immunotherapy of cancer (SITC 2018): Washington DC, USA. 7-11 November 2018. *J Immunother Cancer.* (2018) 6:114. doi: 10.1186/s40425-018-0423-x
201. Aggarwal C, Cohen RB, Morrow MP, Kraynyak KA, Sylvester AJ, Cheung J, et al. Immune therapy targeting E6/E7 oncogenes of human paillomavirus type 6 (HPV-6) reduces or eliminates the need for surgical intervention in the treatment of HPV-6 associated recurrent respiratory papillomatosis. *Vaccines.* (2020) 8:56. doi: 10.3390/vaccines8010056
202. Diehl MC, Lee JC, Daniels SE, Tebas P, Khan AS, Giffear M, et al. Tolerability of intramuscular and intradermal delivery by CELLECTRA((R)) adaptive constant current electroporation device in healthy volunteers. *Hum Vaccin Immunother.* (2013) 9:2246–52. doi: 10.4161/hv.24702
203. Hasan Y, Furtado L, Tergas A, Lee N, Brooks R, McCall A, et al. A Phase I trial assessing the safety and tolerability of a therapeutic DNA vaccination against HPV16 and HPV18 E6/E7 oncogenes after chemoradiation for cervical cancer. *Int J Radiat Oncol Biol Phys.* (2020) 107:487–98. doi: 10.1016/j.ijrobp.2020.02.031
204. Richner JM, Himansu S, Dowd KA, Butler SL, Salazar V, Fox JM, et al. Modified mRNA vaccines protect against zika virus infection. *Cell.* (2017) 168:1114–25.e10. doi: 10.1016/j.cell.2017.02.017
205. Espeseth AS, Cejas PJ, Citron MP, Wang D, DiStefano DJ, Callahan C, et al. Modified mRNA/lipid nanoparticle-based vaccines expressing respiratory syncytial virus F protein variants are immunogenic and protective in rodent models of RSV infection. *NPJ Vaccines.* (2020) 5:16. doi: 10.1038/s41541-020-0163-z
206. Adams D, Gonzalez-Duarte A, O'Riordan WD, Yang CC, Ueda M, Kristen AV, et al. Patisiran, an RNAi therapeutic, for hereditary transthyretin amyloidosis. *N Engl J Med.* (2018) 379:11–21. doi: 10.1056/NEJMoa1716153
207. Stadler CR, Bahr-Mahmud H, Celik L, Hebich B, Roth AS, Roth RP, et al. Elimination of large tumors in mice by mRNA-encoded bispecific antibodies. *Nat Med.* (2017) 23:815–7. doi: 10.1038/nm.4356
208. Beissert T, Perkovic M, Vogel A, Erbar S, Walzer KC, Hempel T, et al. A Trans-amplifying RNA vaccine strategy for induction of potent protective immunity. *Mol Ther.* (2020) 28:119–28. doi: 10.1016/j.ymthe.2019.09.009
209. Sahin U, Kariko K, Tureci O. mRNA-based therapeutics–developing a new class of drugs. *Nat Rev Drug Discov.* (2014) 13:759–80. doi: 10.1038/nrd4278
210. Stensballe LG, Nante E, Jensen IP, Kofoed PE, Poulsen A, Jensen H, et al. Acute lower respiratory tract infections and respiratory syncytial virus in infants in Guinea-Bissau: a beneficial effect of BCG vaccination for girls community based case-control study. *Vaccine.* (2005) 23:1251–7. doi: 10.1016/j.vaccine.2004.09.006

211. Moorlag S, Arts RJW, van Crevel R, Netea MG. Non-specific effects of BCG vaccine on viral infections. *Clin Microbiol Infect.* (2019) 25:1473–8. doi: 10.1016/j.cmi.2019.04.020
212. Kaufmann E, Sanz J, Dunn JL, Khan N, Mendonca LE, Pacis A, et al. BCG educates hematopoietic stem cells to generate protective innate immunity against tuberculosis. *Cell.* (2018) 172:176–90.e19. doi: 10.1016/j.cell.2017.12.031
213. Rauch S, Jasny E, Schmidt KE, Petsch B. New vaccine technologies to combat outbreak situations. *Front Immunol.* (2018) 9:1963. doi: 10.3389/fimmu.2018.01963

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Public Health

OPINION
published: 21 July 2020
doi: 10.3389/fpubh.2020.00397

Coronavirus (SARS-CoV-2) and Mortality Rate in India: The Winning Edge

*Gyaneshwer Chaubey**

Cytogenetics Laboratory, Department of Zoology, Banaras Hindu University, Varanasi, India

Keywords: COVID-19, India, endogamy, disease susceptibility, ACE2, herd immunity

The Covid-19 outbreak is due to a virus which emerged in China at the end of December 2019, and is now widespread in more than 200 countries worldwide (1). Several researches have highlighted that it was introduced to humans from bats (2, 3). The infection spreads like a chain reaction as one infected person passes this virus to two or three others, who then continue to spread it in a similar manner (**Figure 1**). The number of people infected in a region from a single person is estimated as R_0 (R zero). R_0 is the rate at which new infections stem from a single case (4). R_0 <1 indicates the reduction of cases, whereas $R_0 > 1$ suggests that the number of cases are increasing. The global R_0 value for Covid-19 is estimated to range between 3 and 5, which is twice as fast as SARS (Severe Acute Respiratory Syndrome) (5). This is why the spread of Covid-19 is so rapid, and why the number of infected people double every 5–10 days (Coronavirus in India). The socioeconomic impact of Covid-19 is disruptive, and the whole world is looking forward to the end of this crisis. Similar to other countries, its transmission among the Indian population is evident. But the major question is its fate in India, as India makes up one-fifth of the world's population. The recent report makes India's fate more vulnerable, by estimating that the total number of reported cases are 10-fold less than the total number of infected people (6). Thus, such complexity makes India one of the most monitored countries during this pandemic.

Contrary to what one would expect, the emerging data suggests that at the front of mortality, the situation in India turned out not to be as bad as in some European nations and the USA. However, given the fact that India's emergency services are limited, it is likely to be more vulnerable to this pandemic. Keeping this in mind, the Indian government has introduced unprecedented measures (including the stringent and early nation-wide lockdown from March 22), to stop the spread of Covid-19. Wherever a high number of cases are found, it is considered as a hotspot. The locality is immediately sealed to stop the spread of the virus. Further, in lockdown 3.0, the country was divided in to green, orange, and red zones, based on severity and the number of cases (MOHFW-GoI)[1]. However, we are not sure how long this measure should be implemented and what the chances of resurgence will be when these restrictions are relaxed after a few weeks. Equally at the research front, scientists from all over the world are trying to find a way to exit from this crisis. More than 20,000 research papers (doubling every 20 days) on this topic itself suggests its seriousness (7). Indeed, social distancing and other governmental measures would reduce the virus's ability to sweep through the population, but unless, or until a vaccine is discovered, what measures can India rely upon to control the spread of the virus?

The one and most prominent way is to obtain "herd immunity" (8). When a population is exposed to any infectious disease, many of its inhabitants gain immunity in a short period of time. When ∼70% of individuals in the population become immune, this facilitates herd immunity. This barrier of immunity blocks the virus from taking hold and infecting others. Immunity may be sustained for almost a year, and such a time period can buy us time to develop a vaccine. Moreover, immune people can volunteer for the healthcare services and other necessary activities without any sophisticated protective gear. This seems easy to implement, but when looking fatalities figures for

[1] Available at: mohfw.gov.in.

Edited by:
Zisis Kozlakidis, International Agency for Research on Cancer (IARC), France

Reviewed by:
Alok Tiwari, Indian Institute of Technology Bombay, India
Jees Sebastian, The State University of New Jersey, United States

***Correspondence:**
Gyaneshwer Chaubey
gyaneshwer.chaubey@bhu.ac.in

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Public Health

Received: *20 April 2020*
Accepted: *06 July 2020*
Published: *21 July 2020*

Citation:
Chaubey G (2020) Coronavirus (SARS-CoV-2) and Mortality Rate in India: The Winning Edge. Front. Public Health 8:397. doi: 10.3389/fpubh.2020.00397

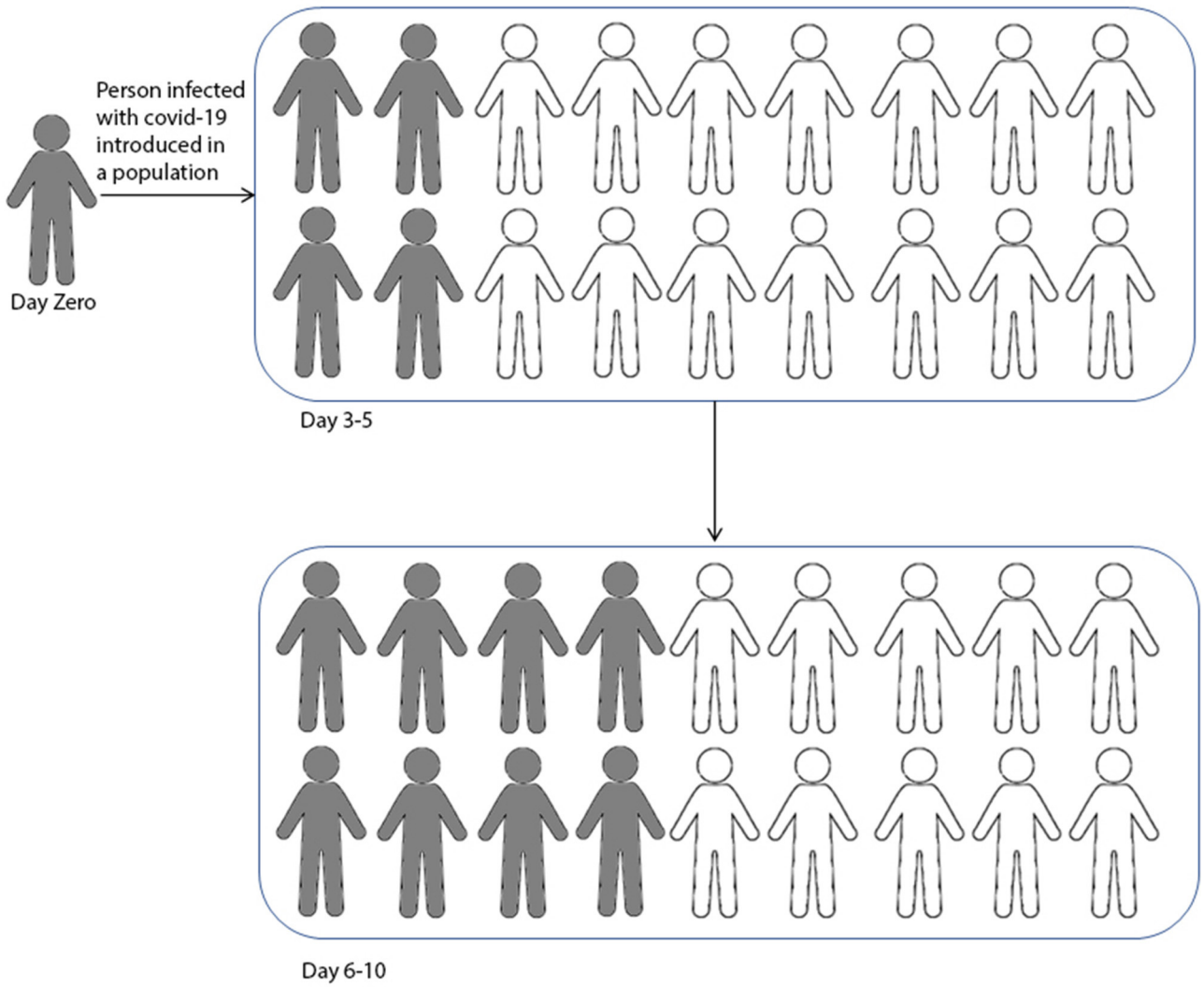


FIGURE 1 | The graphical representation of Covid-19 infection in a natural population. The viral infection spread from peer-to-peer like a chain reaction. This figure is only a graphical representation of the spread, without considering whether someone in the population has recovered or died.

the coronavirus, we cannot put the life of ∼3% (or even more) people on the line (**Figure 2A**). Therefore, thinking of obtaining rapid herd immunity in this particular case, is an autophagy for a nation like India. A recent example is the UK, which had initiated this method in the beginning as a measure, but seeing the severe impact of the virus, abandoned this plan (9, 10).

Accumulating research in other regions of the world are suggesting the effect of high temperatures and humidity on Covid-19 (11–13). However, so far, none of the dedicated studies in India have been performed on the association of temperature or humidity on the spread of Covid-19. Therefore, the relevance of temperature on the spread of Covid-19 in India is not known.

Recent research suggests that the coronavirus receptor of human *ACE2* plays a pivotal role in disease predisposition, therefore, certain polymorphisms in this gene may affect the susceptibility of a population (14–21). As an expert on Human Evolutionary Genetics, it is necessary to reiterate that, in India, modern humans have been living for at least 50,000 to 70,000 years and have experienced various kinds of pathogen pressures (22–25). A large number of genetic and archaeological studies are consistent with a largely local emergence of South Asian ancestry with minor [and in some cases relatively higher e.g., Tibeto-Burmans (26), Austroasiatics (27), and some Northwest Indian populations (28)], ancestry contributions from East and West Eurasians respectively (29–31). Therefore, these long term geographic and genetic isolations, might have certainly helped us to modify our genetic landscape against various kinds of pathogens (22, 25, 32–34). Moreover, the high level of endogamy practices among caste and tribal populations (29, 35) has created a unique genetic profile, and thus, likely variations in *ACE2*. Therefore, it is likely that many of the endogamous populations might have developed a varied degree of susceptibility responses against this virus.

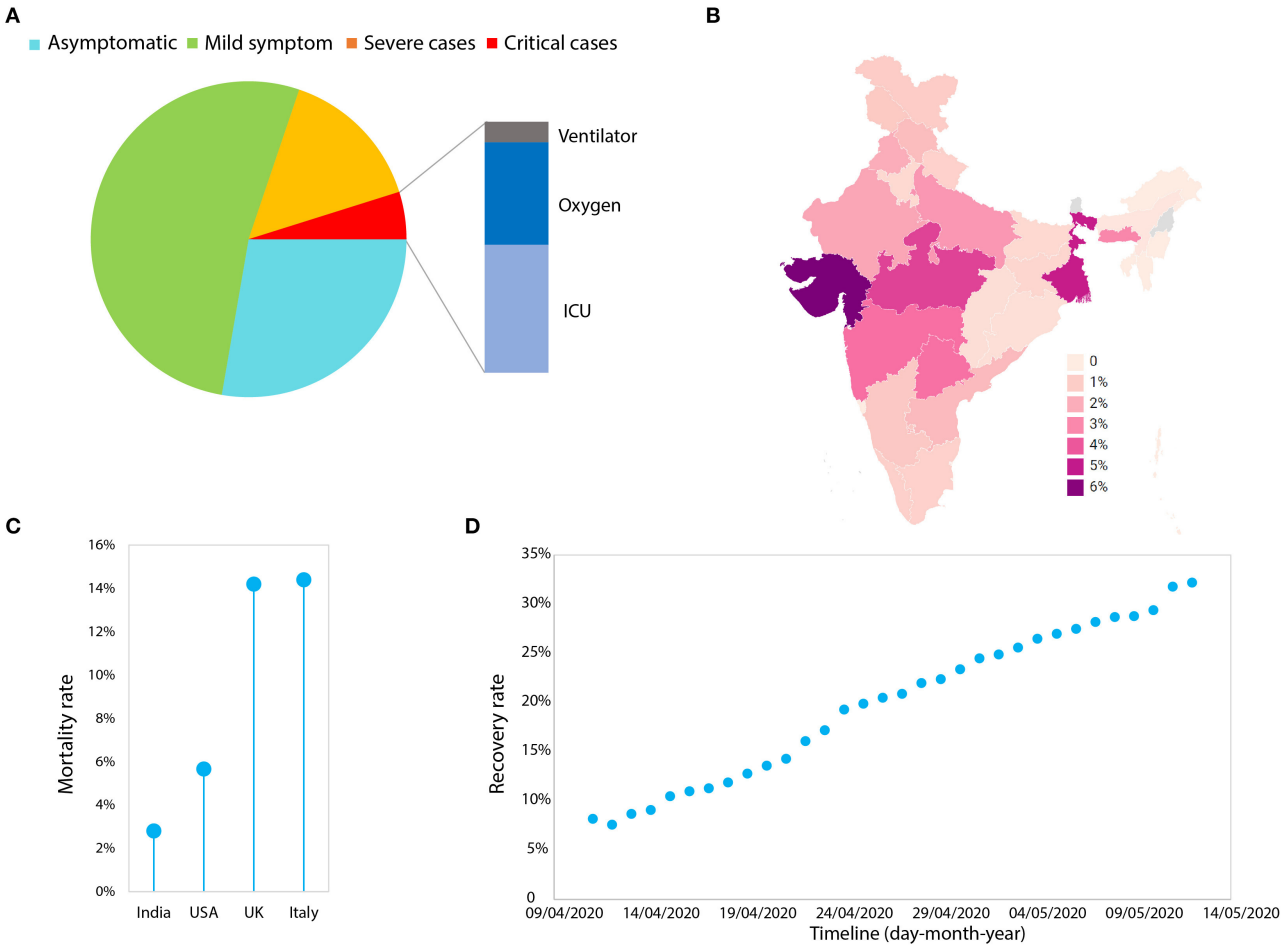


FIGURE 2 | The analysis of Covid-19 from India. **(A)** The frequency (%) of infected people with various levels of symptoms. The data was obtained from (https://www.mohfw.gov.in/). **(B)** The statewide mortality rate (number of people deceased/total number of reported cases) of the infected people. The data was obtained from the https://www.covid19india.org/ and plotted through (https://app.datawrapper.de/). **(C)** The mortality rate of India in comparison with the USA, the UK, and Italy. **(D)** The plot of day-by-day recovery rates (number of people recovered/total number of reported cases) in India (last updated: 12th May 2020). The data was obtained from (https://www.mohfw.gov.in/).

In fact, Cao et al. (15) have looked at the binding sites of the S protein of coronavirus but did not find any variation among 1,000 genome populations. However, keeping in mind that 1,000 genome South Asian samples do not capture the complete South Asian diversity (36), one should look at these variations as well as whole *ACE2* variations in the large number of South Asian ethnic groups. Moreover, the greater role of *ACE2* in disease manifestation is evident in our recent study, which showed that the major South Asian *ACE2* haplotypes are identity by descent (IBD) of East Eurasians rather than West Eurasian (19), which is possibly one of the reasons for the low mortality among the Indian population. Therefore, studying the detailed *ACE2* variations among diverse Indian populations would be worthwhile to understand human susceptibility to Covid-19.

A study done by the Chinese Centre for Disease Control and Prevention on 72,314 cases suggested the presence of 1% asymptomatic individuals (Chinese Center for Disease Control and Prevention)[2]. Asymptomatic individuals are people who have been diagnosed to have a positive viral load but lack any characteristic symptoms including fever, dry cough, and fatigue etc. Recent data in India has identified 28% asymptomatic people—which is alarming (**Figure 2A**). Notably, ∼65% of the Indian population is under the age of 35 years, thus the number of asymptomatic people would likely be much higher than reported (6). Therefore, it is highly alarming and brings to the forefront the question—how can one stop infections that are spread by asymptomatic people?

To identify asymptomatic people, two important dependent measures can be applied. First is mass antigen/antibody testing, and second is to look at their *ACE2* variations. In order to investigate the real spread estimation (6), as

[2]http://www.chinacdc.cn/en/COVID19/ (accessed March 29, 2020).

well as disease spread due to asymptomatic people, the government could initiate, first, mass antigen/antibody testing in containment zones, and second, to see if these people have certain *ACE2* polymorphisms in common. In a positive sense, this would not only help in identifying some of the asymptomatic ethnic groups, but could also help the government in substantially reducing infections (37, 38). Additionally, since the comorbidity is consistent all around the globe (39, 40), serious public awareness is required for those at high risk, to reduce the mortality. The statistics of high risk people in India with diseases such as Diabetes-2 (80M) and Cardiovascular diseases (53M) are daunting, and may require urgent attention (41, 42).

The first nation-wide lockdown of 21 days and further lockdowns 2.0, 3.0, and 4.0, was certainly helpful in restricting asymptomatic people. The governmental measures applied so far have substantially reduced the infection rate R_0 (<1.5) with a doubling rate of cases (i.e., cases double every 15 days) for India [MOHFW-GoI[1]; (43)]. The data available to date suggest a mean death rate of 2.8% for India, which includes 86% comorbidity cases (**Figure 2A**). Many of the states are performing well except those states such as West Bengal and Gujarat, where death rates are closer to the USA i.e., >5% (**Figures 2B,C**). In fact, the recovery rate for India has increased from 7.6 to 32% in a month (**Figure 2D**). Additionally it is highly evident that mortality is extremely low compared to western nations (per 100,000 population; 0.2 for India, 26.6 for the USA and 52.1 for the UK) (**Figures 2B,C**), keeping in mind the low healthcare index 154/195 ranking of India (44). The lockdown measures, public awareness campaigns, and social distancing would have all likely contributed to this low mortality rate.

The growing number of cases in India suggests that the peak of infection has not yet occurred. However, if we look closely, 1/3rd of the cases have been accumulating in two cities, Mumbai and Delhi, and ½ of the cases have occurred in four cities (Mumbai, Delhi, Chennai, and Ahmedabad). If a similar situation occurs in another few densely populated cities, things may be not as under control as we are witnessing today. Therefore, each and every region of India has to develop their own prevention models, considering our past experiences in pandemics, literacy, and healthcare availability (45–47).

It is therefore in India's best interest to continue the use of standard protective measures (48, 49), to conduct systematic epidemiological studies in different real time situations, to test various ethnic groups for *ACE2* variations, to perform mass antigen/antibody testing in containment zones, to identify those who are immune, and to continue the lockdown until R_0 reduces to <1. This will provide massive success in this current health crisis. Collectively, it is likely that with India's diverse ethnic groups and governmental measures, it will not be easy for Covid-19 to cause a large number of casualties.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

ACKNOWLEDGMENTS

The author thanks the reviewers and the editor for their constructive suggestions. The author is also thankful to Dr. Chandana Basu for the discussions. GC was supported by Core Research Grant (CRG/2018/001727) of SERB, India and National Geographic Explorer Grant (HJ3-182R-18).

REFERENCES

1. Coronavirus disease (2019). Available online at: https://www.who.int/emergencies/diseases/novel-coronavirus-2019 (accessed May 13, 2020).
2. Lai C-C, Shih T-P, Ko W-C, Tang H-J, Hsueh P-R. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and corona virus disease-2019 (COVID-19): the epidemic and the challenges. *Int J Antimicrob Agents*. (2020) 55:105924. doi: 10.1016/j.ijantimicag.2020.105924
3. Zhou P, Yang X-L, Wang X-G, Hu B, Zhang L, Zhang W, et al. A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature*. (2020) 579:270–3. doi: 10.1038/s41586-020-2012-7
4. Keeling MJ, Grenfell BT. Individual-based perspectives on R0. *J Theor Biol*. (2000) 203:51–61. doi: 10.1006/jtbi.1999.1064
5. Liu Y, Gayle AA, Wilder-Smith A, Rocklöv J. The reproductive number of COVID-19 is higher compared to SARS coronavirus. *J. Travel Med*. (2020) 27:taaa021. doi: 10.1093/jtm/taaa021
6. Pedersen MG, Meneghini M. Quantifying undetected COVID-19 cases and effects of containment measures in Italy. *medRxiv*. (2020). doi: 10.1101/2020.04.07.20057075
7. Brainard J. Scientists are drowning in COVID-19 papers. Can new tools keep them afloat? *Sci AAAS*. (2020) 368:924–5. doi: 10.1126/science.abc7839
8. Anderson RM, May RM. Vaccination and herd immunity to infectious diseases. *Nature*. (1985) 318:323–9. doi: 10.1038/318323a0
9. Hunter DJ. Covid-19 and the stiff upper lip—The pandemic response in the United Kingdom. *N Engl J Med*. (2020) 382:e31. doi: 10.1056/NEJMp2005755
10. Sibony A-L. The UK COVID-19 response: a behavioural irony? *Eur J Risk Regul*. (2020) 11:350–7. doi: 10.1017/err.2020.22
11. Bukhari Q, Jameel Y. Will coronavirus pandemic diminish by summer? *SSRN*. (2020). doi: 10.2139/ssrn.3556998
12. Dong E, Du H, Gardner L. An interactive web-based dashboard to track COVID-19 in real time. *Lancet Infect Dis*. (2020) 20:533–4. doi: 10.1016/S1473-3099(20)30120-1
13. Wang M, Jiang A, Gong L, Luo L, Guo W, Li C, et al. Temperature significant change COVID-19 transmission in 429 cities. *medRxiv*. (2020). doi: 10.1101/2020.02.22.20025791
14. Asselta R, Paraboschi EM, Mantovani A, Duga S. ACE2 and TMPRSS2 variants and expression as candidates to sex and country differences in COVID-19 severity in Italy. *medRxiv*. (2020). doi: 10.1101/2020.03.30.20047878
15. Cao Y, Li L, Feng Z, Wan S, Huang P, Sun X, et al. Comparative genetic analysis of the novel coronavirus (2019-nCoV/SARS-CoV-2) receptor ACE2 in different populations. *Cell Discov*. (2020) 6:1–4. doi: 10.1038/s41421-020-0147-1
16. Chen J, Jiang Q, Xia X, Liu K, Yu Z, Tao W, et al. Individual variation of the SARS-CoV2 receptor ACE2 gene expression and regulation. *Aging Cell*. (2020) e13168. doi: 10.1111/acel.13168. [Epub ahead of print].
17. Gemmati D, Bramanti B, Serino ML, Secchiero P, Zauli G, Tisato V. COVID-19 and individual genetic susceptibility/receptivity: role of ACE1/ACE2 genes, immunity, inflammation and coagulation. might the double X-chromosome in females be protective against SARS-CoV-2 compared

to the single X-chromosome in males? *Int J Mol Sci*. (2020) 21:3474. doi: 10.3390/ijms21103474
18. Khayat AS, de Assumpcao PP, Khayat BCM, Araujo TMT, Batista-Gomes JA, Imbiriba LC, et al. ACE2 polymorphisms as potential players in COVID-19 outcome. *medRxiv*. (2020). doi: 10.1101/2020.05.27.20114843
19. Srivasta A, Pandey RK, Kumar P, Rasalkar AA, Tamang R, Shrivastava P, et al. Most frequent South Asian haplotypes of ACE2 share identity by descent with East Eurasian populations. *Res Square*. (2020). doi: 10.21203/rs.3.rs-27310/v1
20. Stawiski EW, Diwanji D, Suryamohan K, Gupta R, Fellouse FA, Sathirapongsasuti F, et al. Human ACE2 receptor polymorphisms predict SARS-CoV-2 susceptibility. *bioRxiv*. (2020). doi: 10.1101/2020.04.07.024752
21. Strafella C, Caputo V, Termine A, Barati S, Gambardella S, Borgiani P, et al. Analysis of ACE2 genetic variability among populations highlights a possible link with COVID19-related neurological complications. *Genes*. (2020) 11:741. doi: 10.21203/rs.3.rs-28871/v1
22. Mukherjee S, Sarkar-Roy N, Wagener DK, Majumder PP. Signatures of natural selection are not uniform across genes of innate immune system, but purifying selection is the dominant signature. *Proc Natl Acad Sci USA*. (2009) 106:7073–8. doi: 10.1073/pnas.0811357106
23. Barreiro LB, Quintana-Murci L. From evolutionary genetics to human immunology: how selection shapes host defence genes. *Nat Rev Genet*. (2010) 11:17–30. doi: 10.1038/nrg2698
24. Karlsson EK, Kwiatkowski DP, Sabeti PC. Natural selection and infectious disease in human populations. *Nat Rev Genet*. (2014) 15:379–93. doi: 10.1038/nrg3734
25. Pagani L, Lawson DJ, Jagoda E, Mörseburg A, Eriksson A, Mitt M. Genomic analyses inform on migration events during the peopling of Eurasia. *Nature*. (2016) 538:238–42. doi: 10.1038/nature19792
26. Tamang R, Chaubey G, Nandan A, Govindaraj P, Singh VK, Rai N, et al. Reconstructing the demographic history of the Himalayan and adjoining populations. *Hum Genet*. (2018) 137:129–39. doi: 10.1007/s00439-018-1867-2
27. Tätte K, Pagani L, Pathak AK, Kõks S, Duy BH, Ho XD, et al. The genetic legacy of continental scale admixture in Indian Austroasiatic speakers. *Sci Rep*. (2019) 9:3818. doi: 10.1038/s41598-019-40399-8
28. Pathak AK, Kadian A, Kushniarevich A, Montinaro F, Mondal M, Ongaro L, et al. The genetic ancestry of modern indus valley populations from Northwest India. *Am J Hum Genet*. (2018) 103:918–29. doi: 10.1016/j.ajhg.2018.10.022
29. Chaubey G, Metspalu M, Kivisild T, Villems R. Peopling of South Asia: investigating the caste-tribe continuum in India. *Bioessays News Rev Mol Cell Dev Biol*. (2007) 29:91–100. doi: 10.1002/bies.20525
30. Petraglia M, Clarkson C, Boivin N, Haslam M, Korisettar R, Chaubey G, et al. Population increase and environmental deterioration correspond with microlithic innovations in South Asia ca. 35,000 years ago. *Proc Natl Acad Sci USA*. (2009) 106:12261–6. doi: 10.1073/pnas.0810842106
31. Shinde V, Narasimhan VM, Rohland N, Mallick S, Mah M, Lipson M, et al. An ancient harappan genome lacks ancestry from steppe pastoralists or iranian farmers. *Cell*. (2019). 179:729–35.e10. doi: 10.1016/j.cell.2019.08.048
32. Du Z, Sharma SK, Spellman S, Reed EF, Rajalingam R. KIR2DL5 alleles mark certain combination of activating KIR genes. *Genes Immun*. (2008) 9:470–80. doi: 10.1038/gene.2008.39
33. Pitchappan R, Kavitha V, Jayalakshmi M. HLA genomic diversity of India and its implications in HIV pandemic. *Int J Hum Genet*. (2008) 8:143–53. doi: 10.1080/09723757.2008.11886026
34. Das R, Ghate SD. Investigating the likely association between genetic ancestry and COVID-19 manifestation. *medRxiv*. (2020). doi: 10.1101/2020.04.05.20054627
35. Nakatsuka N, Moorjani P, Rai N, Sarkar B, Tandon A, Patterson N, et al. The promise of discovering population-specific disease-associated genes in South Asia. *Nat Genet*. (2017) 49:1403. doi: 10.1038/ng.3917
36. Sengupta D, Choudhury A, Basu A, Ramsay M. Population stratification and underrepresentation of indian subcontinent genetic diversity in the 1000 genomes project dataset. *Genome Biol Evol*. (2016) 8:3460–70. doi: 10.1093/gbe/evw244
37. Day M. Covid-19: identifying and isolating asymptomatic people helped eliminate virus in Italian village. *BMJ*. (2020) 368:m1165. doi: 10.1136/bmj.m1165
38. Oran DP, Topol EJ. Prevalence of asymptomatic SARS-CoV-2 infection: a narrative review. *Ann Intern Med*. (2020) M20-3012. doi: 10.7326/M20-3012. [Epub ahead of print].
39. Wang T, Du Z, Zhu F, Cao Z, An Y, Gao Y, et al. Comorbidities and multi-organ injuries in the treatment of COVID-19. *Lancet*. (2020) 395:e52. doi: 10.1016/S0140-6736(20)30558-4
40. Yang J, Zheng Y, Gou X, Pu K, Chen Z, Guo Q, et al. Prevalence of comorbidities in the novel Wuhan coronavirus (COVID-19) infection: a systematic review and meta-analysis. *Int J Infect Dis*. (2020) 94:91–5. doi: 10.1016/j.ijid.2020.03.017
41. Anjana RM, Deepa M, Pradeepa R, Mahanta J, Narain K, Das HK, et al. Prevalence of diabetes and prediabetes in 15 states of India: results from the ICMR–INDIAB population-based cross-sectional study. *Lancet Diabetes Endocrinol*. (2017) 5:585–96. doi: 10.1016/S2213-8587(17)30174-2
42. Abdul-Aziz AA, Desikan P, Prabhakaran D, Schroeder LF. Tackling the burden of cardiovascular diseases in India: The essential diagnostics list. *Circ Cardiovasc Qual Outcomes*. (2019) 12:e005195. doi: 10.1161/CIRCOUTCOMES.118.005195
43. Tiwari A. Modelling and analysis of COVID-19 epidemic in India. *medRxiv*. (2020). doi: 10.1101/2020.04.12.20062794
44. Reddy KS, Patel V, Jha P, Paul VK, Kumar AS, Dandona L, et al. Towards achievement of universal health care in India by 2020: a call to action. *Lancet*. (2011) 377:760–8. doi: 10.1016/S0140-6736(10)61960-5
45. Mishra AC, Chadha MS, Choudhary ML, Potdar VA. Pandemic influenza (H1N1) 2009 is associated with severe disease in India. *PLoS ONE*. (2010) 5:e10540. doi: 10.1371/journal.pone.0010540
46. Chandra S, Kassens-Noor E. The evolution of pandemic influenza: evidence from India, 1918–19. *BMC Infect Dis*. (2014) 14:510. doi: 10.1186/1471-2334-14-510
47. Swetha G, Eashwar V, Gopalakrishnan S. Epidemics and pandemics in India throughout history: a review article. *Indian J Public Health Res Dev*. (2019) 10:1570–6. doi: 10.5958/0976-5506.2019.02328.3
48. Singh R, Adhikari R. Age-structured impact of social distancing on the COVID-19 epidemic in India. *arXiv preprints*. arXiv200312055 (2020).
49. Zhang R, Li Y, Zhang AL, Wang Y, Molina MJ. Identifying airborne transmission as the dominant route for the spread of COVID-19. *Proc Natl Acad Sci USA*. (2020) 117:14857–14863. doi: 10.1073/pnas.2009637117

Conflict of Interest: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 21 July 2020
doi: 10.3389/fcimb.2020.00404

Association Between ABO Blood Group System and COVID-19 Susceptibility in Wuhan

Qian Fan[1†], *Wei Zhang*[1†], *Bo Li*[2,3†], *De-Jia Li*[4], *Jian Zhang*[4] *and Fang Zhao*[1*]

[1] Department of Cardiology, Zhongnan Hospital of Wuhan University, Wuhan University, Wuhan, China, [2] The State Key Laboratory Breeding Base of Basic Science of Stomatology (Hubei-MOST) & Key Laboratory of Oral Biomedicine Ministry of Education, School and Hospital of Stomatology, Wuhan University, Wuhan, China, [3] Department of Oral Radiology, School and Hospital of Stomatology, Wuhan University, Wuhan, China, [4] Department of Preventive Health, School of Health Sciences, Wuhan University, Wuhan, China

OPEN ACCESS

Edited by:
Slobodan Paessler,
The University of Texas Medical Branch at Galveston, United States

Reviewed by:
Dohun Pyeon,
Michigan State University, United States
Kun Zhang,
Virginia Commonwealth University, United States

***Correspondence:**
Fang Zhao
cedilanid@163.com

[†]These authors have contributed equally to this work and share first authorship

Specialty section:
This article was submitted to Virus and Host, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: 07 April 2020
Accepted: 30 June 2020
Published: 21 July 2020

Citation:
Fan Q, Zhang W, Li B, Li D-J, Zhang J and Zhao F (2020) Association Between ABO Blood Group System and COVID-19 Susceptibility in Wuhan. Front. Cell. Infect. Microbiol. 10:404. doi: 10.3389/fcimb.2020.00404

Background: The ABO blood group system has been associated with multiple infectious diseases, including hepatitis B, dengue haemorrhagic fever and so on. Coronavirus disease 2019 (COVID-19) is a new respiratory infectious disease and the relationship between COVID-19 and ABO blood group system needs to be explored urgently.

Methods: A hospital-based case-control study was conducted at Zhongnan Hospital of Wuhan University from 1 January 2020 to 5 March 2020. A total of 105 COVID-19 cases and 103 controls were included. The blood group frequency was tested with the chi-square statistic, and odds ratios (ORs) with 95% confidence intervals (CIs) were calculated between cases and controls. In addition, according to gender, the studied population was divided into two subgroups, and we assessed the association between cases and controls by gender. Finally, considering lymphopenia as a feature of COVID-19, the relationship between the ABO blood group and the lymphocyte count was determined in case samples.

Results: The frequencies of blood types A, B, AB, and O were 42.8, 26.7, 8.57, and 21.9%, respectively, in the case group. Association analysis between the ABO blood group and COVID-19 indicated that there was a statistically significant difference for blood type A ($P = 0.04$, OR = 1.33, 95% CI = 1.02–1.73) but not for blood types B, AB or O ($P = 0.48$, OR = 0.90, 95% CI = 0.66–1.23; $P = 0.61$, OR = 0.88, 95% CI = 0.53–1.46; and $P = 0.23$, OR = 0.82, 95% CI = 0.58–1.15, respectively). An analysis stratified by gender revealed that the association was highly significant between blood type A in the female subgroup ($P = 0.02$, OR = 1.56, 95% CI = 1.08–2.27) but not in the male subgroup ($P = 0.51$, OR = 1.14, 95% CI = 0.78–1.67). The average level of lymphocyte count was the lowest with blood type A in patients, however, compared with other blood types, there was still no significant statistical difference.

Conclusions: Our findings provide epidemiological evidence that females with blood type A are susceptible to COVID-19. However, these research results need to be validated in future studies.

Keywords: ABO blood group system, COVID-19, association analysis, female, lymphocyte count

INTRODUCTION

Coronavirus disease 2019 (COVID-19), also named novel coronavirus pneumonia (NCP), was first reported in Wuhan in December 2019 and then gradually spread throughout the country. By early March 2020, more than 80,000 people were infected, nearly 3,200 of whom died in China. The pneumonia outbreak has become a serious public health event. COVID-19 is caused by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), which is a new member of the coronavirus family. There are currently 7 known coronaviruses that can infect humans, such as severe acute respiratory syndrome (SARS) coronavirus and Middle East respiratory syndrome (MERS) coronavirus. Based on current epidemiological investigations, the incubation period is 1–14 days and typically 3–7 days, but there are also cases in which an incubation period of over 14 days is reported (Wang et al., 2020; Wu and McGoogan, 2020). Individuals are contagious during the incubation period, and asymptomatic infection may also become the source of infection. Respiratory droplets and close contact are the major transmission routes. COVID-19 is clinically characterized by fever, fatigue, and dry cough. In severe cases, affected individuals can undergo acute respiratory distress syndrome, septic shock, and even death (Chan et al., 2020; Huang et al., 2020).

The ABO blood group is the most important blood group system in humans and includes 4 blood types, namely, A, AB, B, and O. The human ABO blood group is located on chromosome 9 (9q34.2) (Melzer et al., 2008; Wiggins et al., 2009). Many studies have found that the ABO blood group plays an important role in various human diseases, such as cardiovascular, oncological, and some infectious and non-infectious diseases (Wolpin et al., 2010; Chen et al., 2016). Meanwhile, the system can play a direct role in infection by serving as receptors or coreceptors for microorganisms, parasites, and viruses. Blood group antigens, also named human histo-blood group antigens (HBGAs), are one of the main antigens on the surface of human red blood cells. They represent polymorphic traits inherited among individuals and populations. Differences in blood group antigen expression can increase or decrease host susceptibility to many infections. In addition, many blood group antigens facilitate intracellular uptake, signal transduction, or adhesion through the organization of membrane microdomains and modify the innate immune response to infection (Behal et al., 2010; Singh et al., 2016; Chakrani et al., 2018; Liu et al., 2018).

The ABO blood group has been previously found to contribute to the risk of multiple infectious diseases in a series of studies. Mohammadali et al. reported that the presence of blood group O might significantly decrease the risk of hepatitis B, and the distribution of Rh in HBV-infected individuals was higher between Rh-positive donors (Mohammadali and Pourfathollah, 2014). Elnady et al. found that Rota-positive status for rotavirus gastroenteritis was significantly more prevalent among those with blood type A and significantly less prevalent among those with blood type B (Elnady et al., 2017). Another recent study carried out by Degarege et al. reported that malaria patients with blood group A had a higher risk of anemia than did those with O and non-A phenotypes (Degarege et al., 2012). Among patients infected with dengue virus, Murugananthan et al. found that patients with AB blood had a risk that was more than 2.5 times higher of developing dengue haemorrhagic fever than did those with other blood types (Murugananthan et al., 2018). In addition, a meta-analysis suggested that blood types A, B, and AB might not affect susceptibility to norovirus infection. However, those with blood type O appeared to be more susceptible to this infection (Liao et al., 2020). Because SARS-CoV-2 is a completely new virus, it is unclear whether the ABO blood groups affect individuals' susceptibility to COVID-19.

Hence, we performed a case-control study to explore the relationship between the ABO blood group and COVID-19 in Wuhan and further classified the populations according to gender. Additionally, lymphopenia is a common feature of patients with COVID-19 and might be a critical factor associated with the severity and mortality of the disease (Xu Z. et al., 2020). The association between ABO blood type and the count of lymphocyte was also investigated in cases.

METHODS

Study Design and Data Source

A retrospective case-control association study was performed during the period from 1 January 2020 to 5 March 2020, with a total of 208 subjects (105 cases vs. 103 controls). All subjects were enrolled from Zhongnan Hospital of Wuhan University, which is a hospital designated for the treatment of patients with COVID-19.

All study individuals were subjected to demographics, clinical features, laboratory findings, reports, and chest CT scans. Demographics included age, gender, hypertension, diabetes, and heart disease. Clinical features involved disease manifestations such as fever, cough, dyspnoea, chest tightness, and diarrhea. Laboratory findings included white blood cell count, lymphocyte count, neutrophil ratio, lymphocyte ratio, blood type, and throat swab nucleic acid test results. All information was obtained and analyzed with the standard Excel program. Two doctors independently extracted the data of the eligible individuals, and the results were reviewed by a third investigator.

This study was reviewed and approved by the Medical Ethical Committee of Zhongnan Hospital of Wuhan University. Oral consent was obtained from patients.

Case and Control Selection

The criterion for enrolment as a case was defined according to the Diagnosis and Treatment Scheme for New Coronavirus Pneumonia (Trial version 5, Trial version 6) issued by the General Office of National Health Commission of the People's Republic of China and the Office of State Administration of Traditional Chinese Medicine.

COVID-19 cases were diagnosed as "clinically diagnosed cases" or "confirmed cases" according to the above criteria. The specific diagnostic criteria for clinically diagnosed cases are as follows: (a) history of epidemiology: I Travel history or residence history in Wuhan and surrounding areas within 14 days prior to onset of the disease, or other cases reported in the community; II contact with patients from Wuhan and surrounding areas,

or with fever or respiratory symptoms from the community prior to the onset of the disease, within 14 days prior to onset of the disease; III cluster disease; and IV. contact with a new type of coronavirus infection; (b) clinical manifestations: I fever and/or respiratory symptoms; II imaging features of the above pneumonia; and III normal or decreased total white blood cell count or decreased lymphocyte count at the early stage of onset; and (c) comprehensive evaluation by three COVID-19 consultation experts in the hospital. The specific diagnostic criterion for confirmed cases is as follows: COVID-19 nuclear acid test positive for viral nucleic acid by reverse transcription polymerase chain reaction real-time (RT-PCR) detection with specimens from the respiratory tract or blood samples.

The eligible control subjects were selected from individuals with the following characteristics: (1) gender- and age-matched; (2) no other history of respiratory infections, such as bacterial pneumonia, mycoplasma pneumonia, tuberculosis and other types of pneumonia; (3) no other infectious diseases, such as hepatitis B and AIDS; and (4) no severe liver and kidney dysfunction.

Association Analysis

The association between different blood types and COVID-19 was performed in the selected population. According to gender, subgroups were stratified to assess whether there was a significant difference between blood type and the incidence of COVID-19. In addition, because lymphocyte decline was related to the severity of COVID-19, we performed a correlation analysis between blood group and lymphocyte count in the COVID-19 patients (Chen et al., 2020).

Statistical Analysis

Statistical analysis was carried out using the Statistical Package for Social Sciences (SPSS) version 21.0. Independent sample *t*-tests were used for age, white blood cell count, lymphocyte count, neutrophil ratio, and lymphocyte ratio. A chi-square test was used for hypertension, diabetes, heart disease, tumor, liver disease, and kidney disease. The ABO blood group frequency in all populations and different gender subgroups was tested using chi-square tests and odds ratios (ORs) with 95% confidence intervals (CIs). Analysis of the association between the ABO blood group and the lymphocyte count was performed with analysis of variance (ANOVA) and a linear regression model. A $P < 0.05$ was considered significant.

RESULTS

Distribution of the ABO Blood Group System

Table 1 illustrates the demographic, clinical, and laboratory characteristics of the study population. The present research consisted of 208 participants divided into two groups: the COVID-19 case group and the control group. Of the 105 patients with COVID-19, 55 were males and 50 were females. The age range of patients was 56.8 ± 18.3. The frequencies of blood types A, B, AB, and O were 42.8, 26.7, 8.57, and 21.9%, respectively. In the control group, 56 (54.4%) of the participants were males, and 47 (45.6%) were females. The age range of the control subjects was 54.0 ± 15.0. The distribution of the ABO blood group of the controls was 29.1% for A, 31.1% for B, 29.1% for O and 10.7% for AB.

TABLE 1 | The clinical characteristics of the studied population.

Characteristics	Case	Control	*P*
Number of subjects	105	103	–
Age (years)	56.8 ± 18.3	54.0 ± 15.0	0.228
Gender (male %)	55 (52.4%)	56 (54.4%)	0.774
Hypertension (%)	36 (34.0%)	20 (19.4%)	0.019
Diabetes (%)	11 (10.5%)	9 (8.74%)	0.815
Heart disease (%)	18 (17.1%)	10 (9.71%)	0.155
Tumor (%)	5 (4.76%)	6 (5.83%)	0.767
Liver disease (%)	3 (2.86%)	0 (0.00%)	0.246
Kidney disease (%)	9 (8.57%)	2 (1.94%)	0.06
White blood cell count (10∧9/L)	6.94 ± 3.66	6.27 ± 1.75	0.091
Lymphocyte count (10∧9/L)	0.81 ± 0.47	1.64 ± 0.49	< 0.001
Neutrophil ratio (%)	76.8 ± 13.6	62.3 ± 10.1	< 0.001
Lymphocyte ratio (%)	14.4 ± 10.5	27.6 ± 9.37	< 0.001

The data are presented as the mean ± standard deviation or a percentage.

TABLE 2 | Association analysis of ABO blood type between COVID-19 cases and controls.

Blood group	Case (%)	Control (%)	χ^2	*P*	OR (95% CI)
A	45 (42.8%)	30 (29.1%)	4.25	0.04	1.33 (1.02–1.73)
B	28 (26.7%)	32 (31.1%)	0.49	0.48	0.90 (0.66–1.23)
AB	9 (8.57%)	11 (10.7%)	0.27	0.61	0.88 (0.53–1.46)
O	23 (21.9%)	30 (29.1%)	1.43	0.23	0.82 (0.58–1.15)

OR, odds ratio after adjustment; CI, confidence interval.

Association Between ABO Blood Group and COVID-19

As shown in **Table 2**, we performed a combined association analysis between ABO blood group and COVID-19, which showed a statistically significant difference in COVID-19 infection among those with blood type A ($P = 0.04$, OR = 1.33, 95% CI = 1.02–1.73) but not blood types B, AB or O ($P = 0.48$, OR = 0.90, 95% CI = 0.66–1.23; P=0.61, OR = 0.88, 95% CI = 0.53–1.46; and $P = 0.23$, OR = 0.82, 95% CI = 0.58–1.15, respectively).

Stratified Analysis by Gender

An additional statistical analysis was performed by dividing the entire population into two subgroups by gender, as shown in **Table 3**. The male group comprises 111 subjects, and the female group includes 97 individuals. The association analysis revealed a significant relation between blood type A and COVID-19 in the female subgroup ($P = 0.02$, OR = 1.56, 95% CI = 1.08–2.27) but not in the male subgroup ($P = 0.51$, OR = 1.14, 95% CI = 0.78–1.67).

TABLE 3 | Gender-stratified analysis of ABO blood type and COVID-19 cases.

Blood group	Male		χ^2	*P*	OR (95% CI)	Female		χ^2	*P*	OR (95% CI)
	Case	Control				Case	Control			
A	21	18	0.44	0.51	1.14 (0.78–1.67)	24	12	5.24	0.02	1.56 (1.08–2.27)
B	17	19	0.12	0.73	0.93 (0.62–1.41)	11	13	0.42	0.52	0.85 (0.53–1.39)
AB	6	5	0.12	0.73	1.13 (0.63–1.98)	3	6	1.32	0.25	0.62 (0.24–1.61)
O	11	14	0.40	0.53	0.86 (0.53–1.40)	12	16	1.19	0.28	0.78 (0.48–1.26)

OR, odds ratio after adjustment; CI, confidence interval.

TABLE 4 | Association analysis between the lymphocyte count and ABO blood type in COVID-19 cases.

Blood grouping	*n*	Mean ± SD (10∧9/L)	95% CI	F	*P*
A	45	0.76 ± 0.48	0.61–0.90		
B	28	0.85 ± 0.52	0.65–1.05		
AB	9	0.83 ± 0.27	0.62–1.03	0.30	0.83
O	23	0.85 ± 0.45	0.60–1.04		

SD, standard deviation; CI, confidence intervals.

In addition, blood types B, AB, and O were not significantly associated in either male or female subgroups ($P > 0.05$).

Association Between Lymphocyte Count and COVID-19

As illustrated in **Table 4** and **Figure 1**, the average lymphocyte count levels of individuals with blood type A were lower than those of individuals with blood types B, AB, and O in the case group ($0.76*10^9$/L, $0.85*10^9$/L, $0.83*10^9$/L and $0.85*10^9$/L, respectively).

Unfortunately, statistical analysis showed that blood type A was not significantly associated with lymphocyte count levels in case subjects ($P = 0.83$, $F = 0.30$).

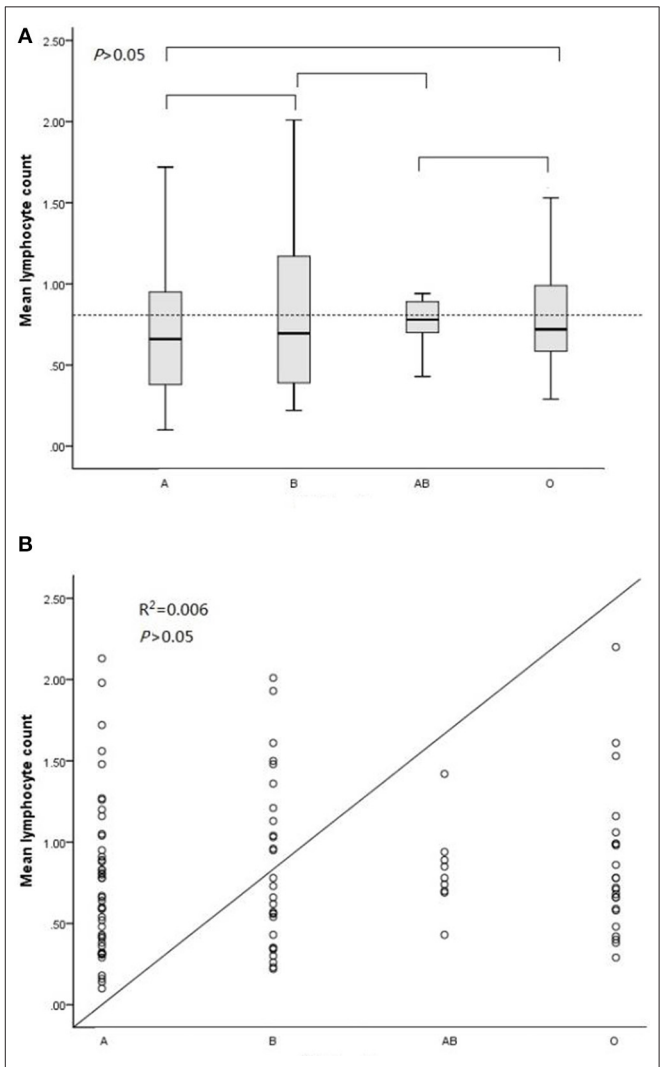


FIGURE 1 | ABO blood type and lymphocyte count level analysis in the case group. **(A)** Comparisons among every pair of the four blood types via ANOVA. **(B)** Association analysis between the lymphocyte count and ABO blood type in a linear regression model.

DISCUSSION

Of the human blood group systems, the ABO blood group is widely used in clinical practice. As some of the important antigens, HBGAs are complex carbohydrate molecules with specific oligosaccharide sequences expressed on the surface of red blood cell membranes. These antigens are also highly expressed on a large number of human cells and tissues, including epithelia, platelets, vascular endothelia and neurons (Storry and Olsson, 2009; Liumbruno and Franchini, 2013; Heggelund et al., 2017; Kazi et al., 2017). HBGAs have been postulated to modify the spread of pathogens through the action of natural antibodies and complements (Neil et al., 2005; Ewald and Sumner, 2018). ABO antibodies are part of the innate immune system against some parasites, bacteria and enveloped viruses, and HBGAs are important as receptors for immune and inflammatory responses (Cooling, 2015; Jing et al., 2020). Meanwhile, this system is often used as a genetic marker in the human genome, generated by a polymorphic glycosyl-transferase encoded by 2 dominant active and a recessive inactive alleles. The association between ABO blood groups and infectious and non-infectious diseases has been widely explored (Groot et al., 2020).

In the current study, we aimed to evaluate the contribution of the ABO blood group to COVID-19 susceptibility in Wuhan by employing a case-control association analysis. Our present results demonstrated that there was a significant association between the A blood group and COVID-19, such that females (but not males) with blood type A were more susceptible to COVID-19 infection. Compared with other patients, female patients with blood type A had a relative risk of 1.33 for coronavirus infection. Xiong et al. recently also found that women show different characteristics from men in the transmission of COVID-19 (Xiong et al., 2020). We speculate that this result may be related to the different anatomic structures, estrogen levels, immune systems and genetic backgrounds of men and women. We further investigated the possible association between ABO blood group and lymphocyte count, the latter was considered as one of the index to evaluate the severity of COVID-19. Although statistical analysis showed no significant difference in ABO blood group and lymphocyte counts, our study found that the decreased lymphocyte counts in patients with blood type A were lower than those in patients with other blood types. The possible explanation for this finding may be related to the small sample size.

In fact, a number of epidemiological studies had also been conducted. For instance, the study of Li et al. reported that the proportion of blood type A in patients infected with SARS-CoV-2 was significantly higher than that in healthy controls (0.38 vs. 0.32%, $P < 0.001$), while the proportion of blood type O in SARS-CoV-2 infected patients was significantly lower than in healthy controls (0.26 vs. 0.34%, $P < 0.001$) (Li et al., 2020). In another study, Zhao et al. also showed that blood type A was associated with an increased risk of SARS-CoV-2 infection, whereas blood type O was associated with a decreased risk (Gerard et al., 2020; Zhao et al., 2020). The main finding of our study was consistent with the above analysis by Li et al. and Zhao et al., but slightly different. In our cases, the relationship between ABO blood type and the count of lymphocyte was further investigated, due to the importance of lymphocyte count in the evaluation of severity of COVID-19.

As with COVID-19, SARS is also a serious respiratory infectious disease. Nevertheless, ABO blood group-associated susceptibility to SARS is different from the corresponding susceptibility to COVID-19. In 2005, Cheng et al. found that individuals with blood type O had a reduced susceptibility to SARS infection in the Hong Kong population. Variable binding affinity to differing ABH substances present in gut epithelial cells may be the cause of the above phenomenon (Cheng et al., 2005).

SARS-CoV-2 belongs to lineage B betacoronavirus and shares high sequence identity with that of bat or human severe acute respiratory syndrome coronavirus-related coronavirus (SARSr-CoV) (Tian et al., 2020). The structural analysis of SARS-CoV-2 contains two important viral proteins, the nucleocapsid and the spike (S) proteins. S proteins of coronaviruses are large transmembrane heavily N-glycosylated proteins that mediate association with a cell surface receptor. SARS-CoV-2 makes use of the S protein to gain entry into the host (Li et al., 2006; Wrapp et al., 2020). Angiotensin-converting enzyme 2 (ACE2) is the main host cell receptor of SARS-CoV-2 and plays a crucial role in the entry of the virus into the cell to cause the final infection (Cao et al., 2020; Wu, 2020; Xu H. et al., 2020). The relationship between natural antibodies of the ABO blood system and the ACE2 interaction has been experimentally investigated. In 2008, Guillon et al. observed that S protein/ACE2-dependent adhesion of special Chinese hamster ovary cells to an ACE2-expressing cell line could be specifically inhibited by either monoclonal or human natural anti-A antibodies. Their findings indicated that anti-A antibodies may block the interaction between the SARS coronavirus and its receptor-ACE2, thereby providing protection (Guillon et al., 2008). This is consistent with our findings, suggesting that those with blood type A may be more susceptible to viral infection.

Meanwhile, several drawbacks existed in our study. First, Due to the limited sample size of COVID-19 in the early stages, the sample size included in our research is not very large. Second, regional selection bias needs to be considered. Third, other potential diseases may affect the research results. Finally, some of the control individuals might develop COVID-19 in the future.

In conclusion, female patients with blood type A are susceptible to COVID-19 in Wuhan after gender stratification. However, more studies are necessary to confirm these findings in a larger sample and among individuals of different ethnicities. The underlying mechanism between the ABO blood groups and ACE2 needs to be further explored.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Medical Ethics Committee, Zhongnan Hospital of Wuhan University. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

FZ and QF had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. QF, D-JL, and JZ performed statistical analysis. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the Chinese Universities Scientific Fund (No. 2042018kf0136) to QF and Scientific Research Project of Hubei Provincial Health Committee (WJ2019M208) to FZ.

REFERENCES

Behal, R., Jain, R., Behal, K. K., and Dhole, T. N. (2010). Variation in the host ABO blood group may be associated with susceptibility to hepatitis C virus infection. *Epidemiol. Infect.* 138, 1096–1099. doi: 10.1017/S0950268809991117

Cao, Y., Li, L., Feng, Z., Wan, S., Huang, P., Sun, X., et al. (2020). Comparative genetic analysis of the novel coronavirus (2019-nCoV/SARS-CoV-2) receptor ACE2 in different populations. *Cell Discov.* 6:11. doi: 10.1038/s41421-020-0147-1

Chakrani, Z., Robinson, K., and Taye, B. (2018). Association between ABO blood groups and *Helicobacter pylori* infection: a meta-analysis. *Sci. Rep.* 8:17604. doi: 10.1038/s41598-018-36006-x

Chan, J. F., Yuan, S., Kok, K. H., To, K. K., Chu, H., Yang, J., et al. (2020). A familial cluster of pneumonia associated with the 2019 novel coronavirus indicating person-to-person transmission: a study of a family cluster. *Lancet* 395, 514–523. doi: 10.1016/S0140-6736(20)30154-9

Chen, N., Zhou, M., Dong, X., Qu, J., Gong, F., Han, Y., et al. (2020). Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study. *Lancet* 395, 507–513. doi: 10.1016/S0140-6736(20)30211-7

Chen, Z., Yang, S. H., Xu, H., and Li, J. J. (2016). ABO blood group system and the coronary artery disease: an updated systematic review and meta-analysis. *Sci. Rep.* 6:23250. doi: 10.1038/srep23250

Cheng, Y., Cheng, G., Chui, C. H., Lau, F. Y., Chan, P. K., Ng, M. H., et al. (2005). ABO blood group and susceptibility to severe acute respiratory syndrome. *JAMA* 293, 1450–1451. doi: 10.1001/jama.293.12.1450-c

Cooling, L. (2015). Blood Groups in Infection and Host Susceptibility. *Clin. Microbiol. Rev.* 28, 801–870. doi: 10.1128/CMR.00109-14

Degarege, A., Medhin, G., Animut, A., Legess, M., and Erko, B. (2012). Association of ABO blood group and P. falciparum malaria related outcomes: a cross-sectional study in Ethiopia. *Acta Trop.* 123, 164–169. doi: 10.1016/j.actatropica.2012.04.012

Elnady, H. G., Abdel, S. O., Saleh, M. T., Sherif, L. S., Abdalmoneam, N., Kholoussi, N. M., et al. (2017). ABO blood grouping in Egyptian children with rotavirus gastroenteritis. *Prz. Gastroenterol.* 12, 175–180. doi: 10.5114/pg.2017.70469

Ewald, D. R., and Sumner, S. (2018). Human microbiota, blood group antigens, and disease. *Wiley Interdiscip. Rev. Syst. Biol. Med.* 10:e1413. doi: 10.1002/wsbm.1413

Gerard, C., Maggipinto, G., and Minon, J. M. (2020). COVID-19 and ABO blood group: another viewpoint. *Br. J. Haematol.* doi: 10.1111/bjh.16884. [Epub ahead of print].

Groot, H. E., Villegas, S. L., Said, M. A., Lipsic, E., Karper, J. C., and van der Harst, P. (2020). Genetically determined ABO blood group and its associations with health and disease. *Arteriosc. Thromb. Vasc. Biol.* 40, 830–838. doi: 10.1161/ATVBAHA.119.313658

Guillon, P., Clement, M., Sebille, V., Rivain, J. G., Chou, C. F., Ruvoen-Clouet, N., et al. (2008). Inhibition of the interaction between the SARS-CoV spike protein and its cellular receptor by anti-histo-blood group antibodies. *Glycobiology* 18, 1085–1093. doi: 10.1093/glycob/cwn093

Heggelund, J. E., Varrot, A., Imberty, A., and Krengel, U. (2017). Histo-blood group antigens as mediators of infections. *Curr. Opin. Struct. Biol.* 44, 190–200. doi: 10.1016/j.sbi.2017.04.001

Huang, C., Wang, Y., Li, X., Ren, L., Zhao, J., Hu, Y., et al. (2020). Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet* 395, 497–506. doi: 10.1016/S0140-6736(20)30183-5

Jing, W., Zhao, S., Liu, J., and Liu, M. (2020). ABO blood groups and hepatitis B virus infection: a systematic review and meta-analysis. *BMJ Open* 10:e034114. doi: 10.1136/bmjopen-2019-034114

Kazi, A. M., Cortese, M. M., Yu, Y., Lopman, B., Morrow, A. L., Fleming, J. A., et al. (2017). Secretor and salivary ABO blood group antigen status predict rotavirus vaccine take in infants. *J. Infect. Dis.* 215, 786–789. doi: 10.1093/infdis/jix028

Li, J., Wang, X., Chen, J., Cai, Y., Deng, A., and Yang, M. (2020). Association between ABO blood groups and risk of SARS-CoV-2 pneumonia. *Br. J. Haematol.* 190, 24–27. doi: 10.1111/bjh.16797

Li, W., Wong, S. K., Li, F., Kuhn, J. H., Huang, I. C., Choe, H., et al. (2006). Animal origins of the severe acute respiratory syndrome coronavirus: insight from ACE2-S-protein interactions. *J. Virol.* 80, 4211–4219. doi: 10.1128/JVI.80.9.4211-4219.2006

Liao, Y., Xue, L., Gao, J., Wu, A., and Kou, X. (2020). ABO blood group-associated susceptibility to norovirus infection: a systematic review and meta-analysis. *Infect. Genet. Evol.* 81:104245. doi: 10.1016/j.meegid.2020.104245

Liu, J., Zhang, S., Liu, M., Wang, Q., Shen, H., and Zhang, Y. (2018). Distribution of ABO/Rh blood groups and their association with hepatitis B virus infection in 3.8 million Chinese adults: a population-based cross-sectional study. *J. Viral. Hepat.* 25, 401–411. doi: 10.1111/jvh.12829

Liumbruno, G. M., and Franchini, M. (2013). Beyond immunohaematology: the role of the ABO blood group in human diseases. *Blood Transfus.* 11, 491–499. doi: 10.2450/2013.0152-13

Melzer, D., Perry, J. R., Hernandez, D., Corsi, A. M., Stevens, K., Rafferty, I., et al. (2008). A genome-wide association study identifies protein quantitative trait loci (pQTLs). *PLoS Genet.* 4:e1000072. doi: 10.1371/journal.pgen.1000072

Mohammadali, F., and Pourfathollah, A. (2014). Association of ABO and Rh blood groups to blood-borne infections among blood donors in Tehran-Iran. *Iran. J. Public Health* 43, 981–989.

Murugananthan, K., Subramaniyam, S., Kumanan, T., Owens, L., Ketheesan, N., and Noordeen, F. (2018). Blood group AB is associated with severe forms of dengue virus infection. *Virusdisease* 29, 103–105. doi: 10.1007/s13337-018-0426-8

Neil, S. J., McKnight, A., Gustafsson, K., and Weiss, R. A. (2005). HIV-1 incorporates ABO histo-blood group antigens that sensitize virions to complement-mediated inactivation. *Blood* 105, 4693–4699. doi: 10.1182/blood-2004-11-4267

Singh, B. K., Leuthold, M. M., and Hansman, G. S. (2016). tructural Constraints on Human Norovirus Binding to Histo-Blood Group Antigens. *mSphere* 1:e00049–16. doi: 10.1128/mSphere.00049-16

Storry, J. R., and Olsson, M. L. (2009). The ABO blood group system revisited: a review and update. *Immunohematology* 25, 48–59.

Tian, X., Li, C., Huang, A., Xia, S., Lu, S., Shi, Z., et al. (2020). Potent binding of 2019 novel coronavirus spike protein by a SARS coronavirus-specific human monoclonal antibody. *Emerg. Microbes Infect.* 9, 382–385. doi: 10.1080/22221751.2020.1729069

Wang, D., Hu, B., Hu, C., Zhu, F., Liu, X., Zhang, J., et al. (2020). Clinical Characteristics of 138 Hospitalized Patients With 2019 Novel Coronavirus-Infected Pneumonia in Wuhan, China. *JAMA.* 323, 1061–1069. doi: 10.1001/jama.2020.1585

Wiggins, K. L., Smith, N. L., Glazer, N. L., Rosendaal, F. R., Heckbert, S. R., Psaty, B. M., et al. (2009). ABO genotype and risk of thrombotic events and hemorrhagic stroke. *J. Thromb. Haemost.* 7, 263–269. doi: 10.1111/j.1538-7836.2008.03243.x

Wolpin, B. M., Kraft, P., Gross, M., Helzlsouer, K., Bueno-de-Mesquita, H. B., Steplowski, E., et al. (2010). Pancreatic cancer risk and ABO blood group alleles: results from the pancreatic cancer cohort consortium. *Cancer Res.* 70, 1015–1023. doi: 10.1158/0008-5472.CAN-09-2993

Wrapp, D., Wang, N., Corbett, K. S., Goldsmith, J. A., Hsieh, C. L., Abiona, O., et al. (2020). Cryo-EM structure of the 2019-nCoV spike in the prefusion conformation. *Science* 367, 1260–1263. doi: 10.1126/science.abb2507

Wu, Y. (2020). Compensation of ACE2 Function for Possible Clinical Management of 2019-nCoV-Induced Acute Lung Injury. *Virol Sin.* 35, 256–258. doi: 10.1007/s12250-020-00205-6

Wu, Z., and McGoogan, J. M. (2020). Characteristics of and Important Lessons From the Coronavirus Disease 2019 (COVID-19) Outbreak in China: Summary of a Report of 72314 Cases From the Chinese Center for Disease Control and Prevention. *JAMA.* 323, 1239–1242. doi: 10.1001/jama.2020.2648

Xiong, Q., Xu, M., Zhang, J., Ji, M., An, P., Lei, H., et al. (2020). Women may play a more important role in the transmission of the Corona virus disease (COVID-19) than men. *MedRxiv [Preprint].*

Xu, H., Zhong, L., Deng, J., Peng, J., Dan, H., Zeng, X., et al. (2020). High expression of ACE2 receptor of 2019-nCoV on the epithelial cells of oral mucosa. *Int. J. Oral Sci.* 12:8. doi: 10.1038/s41368-020-0074-x

Xu, Z., Shi, L., Wang, Y., Zhang, J., Huang, L., Zhang, C., et al. (2020). Pathological findings of COVID-19 associated with acute respiratory distress syndrome. *Lancet Respir. Med.* 8, 420–422. doi: 10.1016/S2213-2600(20)30076-X

Zhao, J., Yang, Y., Huang, H., Li, D., and Gu, D., Lu, X., et al. (2020). Relationship between the ABO blood group and the COVID-19 susceptibility. *MedRxiv*. doi: 10.1101/2020.03.11.20031096. [Epub ahead of print].

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Medicine

POLICY AND PRACTICE REVIEWS
published: 22 July 2020
doi: 10.3389/fmed.2020.00460

Emergency Response Measures for Anesthesia Nursing During the COVID-19 Pandemic: West China Hospital Experiences

Ping Zheng[1,2†], *Ruihao Zhou*[3†], *Lu Yin*[1,2], *Xiaorong Yin*[1,2], *Yongqiao Mao*[1,2], *Heng Wang*[1,2], *Ling Ye*[3*] and *Tao Zhu*[1*]

[1] Department of Anesthesiology, West China Hospital, Sichuan University, Chengdu, China, [2] West China School of Nursing, Sichuan University, Chengdu, China, [3] Department of Pain Management, West China Hospital, Sichuan University, Chengdu, China

OPEN ACCESS

Edited by:
Ata Murat Kaynar,
University of Pittsburgh, United States

Reviewed by:
Wolfgang Weihs,
Medical University of Vienna, Austria
Massimiliano Sorbello,
Gaspare Rodolico Hospital, Italy

***Correspondence:**
Ling Ye
zerodq_hx@163.com
Tao Zhu
xwtao_zhu@yahoo.com

[†] These authors have contributed equally to this work and share first authorship

Specialty section:
This article was submitted to Intensive Care Medicine and Anesthesiology, a section of the journal Frontiers in Medicine

Received: 16 April 2020
Accepted: 09 July 2020
Published: 22 July 2020

Citation:
Zheng P, Zhou R, Yin L, Yin X, Mao Y, Wang H, Ye L and Zhu T (2020) Emergency Response Measures for Anesthesia Nursing During the COVID-19 Pandemic: West China Hospital Experiences. Front. Med. 7:460. doi: 10.3389/fmed.2020.00460

During the COVID-19 pandemic, ensuring the gradual recovery of anesthesia nursing unit and avoiding cross-infection between surgical patients and staff are difficult problems for hospital managers. We outlined the emergency response measures and the transition to normal operation of the anesthesia nursing unit in West China Hospital, which is a large teaching hospital. This mainly included hospital and operating room channel management, three-level screening management of patients and medical staff, classification management of patients undergoing anesthesia and recovery, training management of medical personnel, strict environmental management, and online teaching management.

Keywords: novel coronavirus pneumonia, COVID-19, epidemic prevention, anesthesia nursing, nursing management

INTRODUCTION

A novel coronavirus pneumonia outbreak occurred in Wuhan, Hubei, China, in December 2019 (1, 2). Following more than 2 months of struggle, China's epidemic ultimately began to show a downward trend (3). During the coronavirus disease (COVID-19) pandemic, ensuring the gradual recovery of anesthesia nursing unit and avoiding cross-infection between surgical patients and staff are difficult problems for hospital managers. West China Hospital of Sichuan University, a comprehensive teaching hospital with more than 4,000 beds, is an emergency and critical treatment center in Western China. To effectively meet the needs of daily diagnosis and treatment, from February 10, 2020, the anesthesia nursing unit, as well as the operation of elective surgery, was slowly resumed. According to the operation in the past month, we outlined the epidemic prevention and control strategies for anesthesia nursing units and then strictly implemented them.

From February 10 to March 20, 2020, 2213 patients underwent postoperative anesthesia recovery nursing. No cross-infection occurred in the COVID-19 hospital, and no adverse nursing events took place. During the epidemic period, it was impossible to stop all scheduled operations. Our aim is to help anesthesia and nursing departments worldwide based on our experience of epidemic prevention and control, as will be discussed in the following text.

We statistically evaluated the basic information of the 2,213 patients, such as age, gender, preoperative fever, fever patients with COVID-19 nucleic acid test, preoperative CT, ASA grade, anesthesia method, and operation type. The age frequency distribution of the patients was mainly distributed in the following age groups: 50–59, 40–49, 60–69, and 0–9 years old (**Figure 1A**). Also, the gender distribution (**Figure 1B**) was 1,167 males (52.7%) and 1,046 females (47.3%). The patients were also assessed for preoperative fever ($\geq$37.3°C), and the nucleic acid test results of 13 patients (0.59%) with fever (**Figure 1C**) were negative. The principal distribution (**Figure 1D**) of patients with 3 days preoperative CT was normal (n = 1,598, 72.2%), and increased lung texture (n = 210, 9.5%), COPD (n = 270, 12.3%), and Pulmonary nodules (n = 135, 6.1%) were noted. The ASA grade (**Figure 1E**) was chiefly composed of ASA I (n = 41, 1.9%), ASA II (n = 1,613, 72.9%), ASA (n = 549, 24.8%), and ASA IV (n = 10, 0.5%), and anesthesia methods (**Figure 1F**) were principally intravenous inhalation combined anesthesia (n = 1,893, 85.54%), total intravenous anesthesia (n = 167, 7.55%), and inhalation anesthesia (n = 153, 6.91%); in addition, the top 3 surgical types (**Figure 1G**) were orthopedic surgery (n = 370), gastrointestinal surgery (n = 236), and pediatric surgery (n = 201).

HOSPITAL AND OPERATING ROOM CHANNEL MANAGEMENT

Implementation of Three-Channel Management for Patients in All Buildings

We report the building plan and channel management of the outpatient building, the first inpatient building, and the second inpatient building of West China Hospital (**Figure 2**). According to the hospital outpatient spatial structure, the patient treatment route was converted to one-way, and the entrances and exits were, respectively, arranged at the two ends of the outpatient building. Patients could only enter from the entrance and exit from the exit, and the rest of the access was closed in the meantime.

The implementation of "three-channel" management in all buildings of the hospital meant that the entrances and exits of operation inpatients, inpatients and their escorts, and medical staff were separated and run separately without crossing each other (**Figure 2**). Patients and the person accompanying them were given an admission certificate. Personnel with no certificate were not allowed to enter under strict control. All personnel entering hospital buildings had to wear masks and take temperature measurements properly.

Transformation of Patient Access to the Operating Room

The route of patients entering and leaving the operating room was fixed to one-way transport. Prior to the operation, their temperature was taken thrice in the entrance of the inpatient buildings and operating rooms and also inside the operating rooms. When the temperature was not normal, a report had to be made to the infection management department of the hospital that included the patient's epidemiological history. Experts from the infectious disease department and respiratory department would determine whether the operation could be pushed through as usual following consultation. Following the operation, the patient's tracheal intubation or laryngeal mask had to be removed in the operating room, and then the patients were transferred to the anesthesia recovery room for recuperation. The post-anesthesia care units were on the 10th floor of the first inpatient building, and the operating rooms were on the 11th to 13th floor of the second inpatient building. The two buildings are connected by corridors. If the vital signs of patients were stable and reached the standard of withdrawal from the anesthesia recovery room, the anesthesia nurse would return them to the ward via the "operation inpatient elevator."

THREE-LEVEL SCREENING MANAGEMENT OF PATIENTS AND MEDICAL STAFF

Surgical Patients During Hospitalization

In theory, patients were not allowed to come with an accompanying person. However, in special cases, "one patient, one accompanying person" (one fixed person) was strictly implemented. The head nurse would evaluate the epidemiological history and then issue the accompanying certificate.

A "three-level screening" system was applied in each ward and outlined as follows:

1. First-level screening: Each nursing unit was under the control of a specially assigned person for 24 h, and the patient or/and the accompanying person had to enter with a certificate. The temperature and epidemiological history had to be inquired about and registered.
2. Second-level screening: The patient's temperature had to be monitored as required, and the temperature of the accompanying person had to be monitored thrice a day. The epidemiological history, dizziness, chest tightness, fatigue, and other symptoms had to be inquired about so that people having these problems could be found in time and reported to the infection management department of the hospital.
3. Third-level screening: When the doctor did rounds in the room, the patient would again be asked about his/her epidemiological history and whether he/she had dizziness, chest tightness, and fatigue. Patients had to complete a chest CT examination prior to the operation to check whether they had COVID-19 (4).

Pre-job Epidemiological Screening of Medical Staff

Following the Chinese Lunar New Year holiday, the medical staff needed to report to the department managers their health status, their activity regarding going out and returning during the epidemic, their epidemiological contact history, etc. Before returning to work, those who had left Sichuan Province and returned to Chengdu for <14 days had to isolate at home and not return to work. Until the isolation time reached 14 days following return with no infection symptoms, they needed to

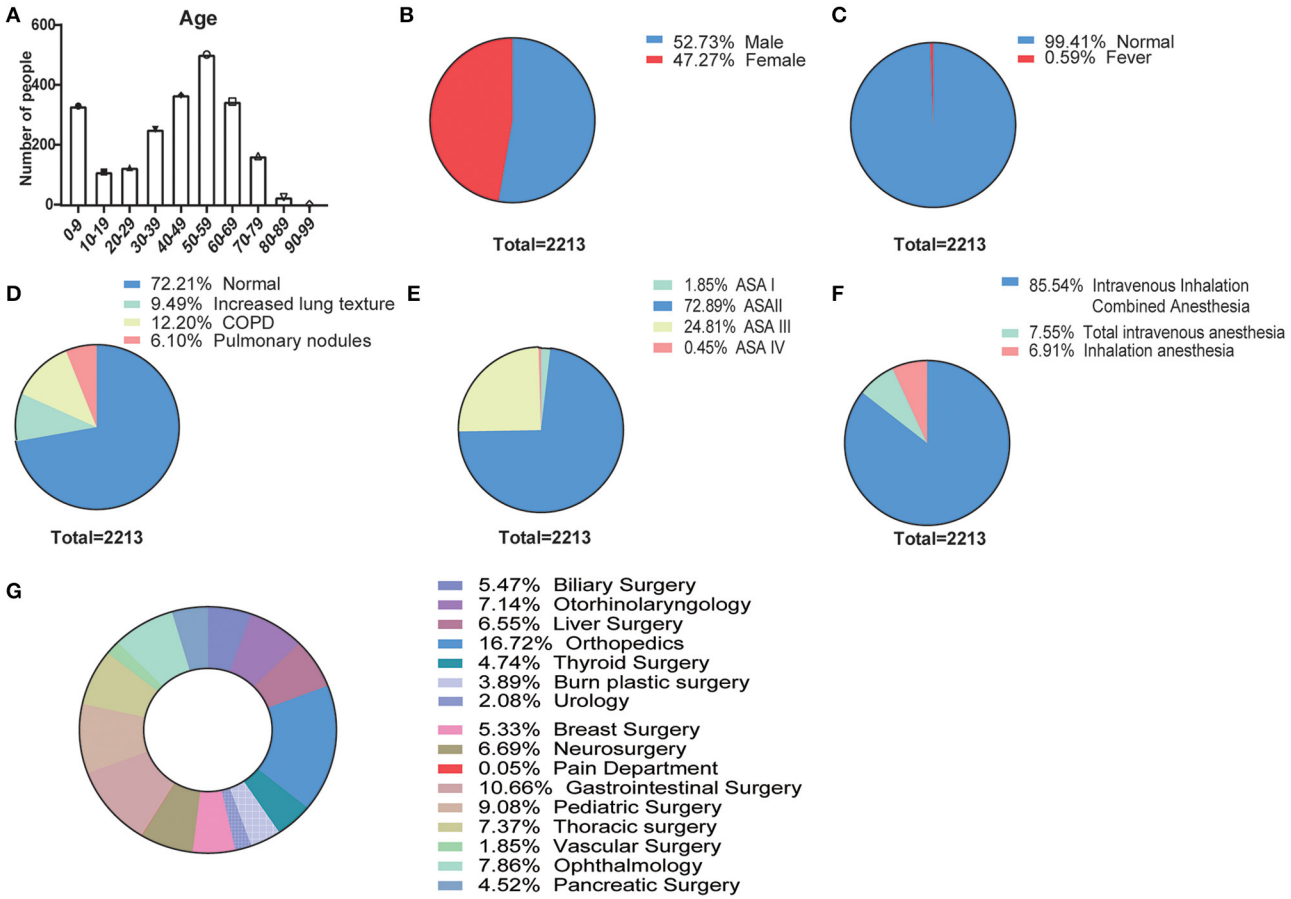


FIGURE 1 | Baseline characteristics of patients who underwent postoperative anesthesia in the COVID-19 pandemic ($N = 2,213$). **(A)** Age frequency distribution of the patients for every 10 years; pie charts of **(B)** gender distribution (male, female), **(C)** preoperative fever (≥37.3°C, normal, fever), **(D)** 3-day preoperative CT (normal, increased lung texture, COPD, pulmonary nodules), **(E)** ASA grade (ASA I, ASA II, ASA III, and ASA IV), **(F)** anesthesia methods (intravenous inhalation combined anesthesia, total intravenous anesthesia, and inhalation anesthesia), and **(G)** surgical types.

apply to the department for a certificate to return to work. Medical staff had to wear masks and have work permits, and a special passage was set up for them before entering and leaving each inpatient building. Temperature measurement and registration were again performed before lunch and on leaving the department after work.

CLASSIFICATION MANAGEMENT OF PATIENTS UNDERGOING ANESTHESIA AND RECOVERY

Special Management of Ordinary Patients

Following the operation, the tracheal intubation or laryngeal mask was removed from the patient in the operating room, and they were then sent to the anesthesia recovery room for further assistance. Not only could this save on medical protective materials, but it also avoided the spread of aerosol in the recovery room due to tracheal intubation and extubation. Following the extubation of the tracheal intubation or laryngeal mask, patients were given low-flow oxygen via a facemask with a reservoir and a medical surgical mask once they were breathing smoothly and were then sent to the anesthesia recovery room.

At the end of each operation, the operating room would be disinfected. In addition to routine anesthesia and care, special attention was paid to ensuring the following:

1. The space between beds of resuscitated patients was >1 meter to lessen the possibility of cross-infection between patients.
2. In the recovery room, patients used a low-flow, non-humidification nasal prong to inhale oxygen, reducing aerosol production.
3. During the recovery period, the patients wore masks and were given low-flow nasal oxygen.
4. The patient's temperature was monitored. If the temperature exceeded 37.3°C, it was reported immediately to the head nurse, the surgeon in charge, and the anesthesiologist during the operation. The causes of fever were assessed in different ways until the possibility of infection with COVID-19 was eliminated.

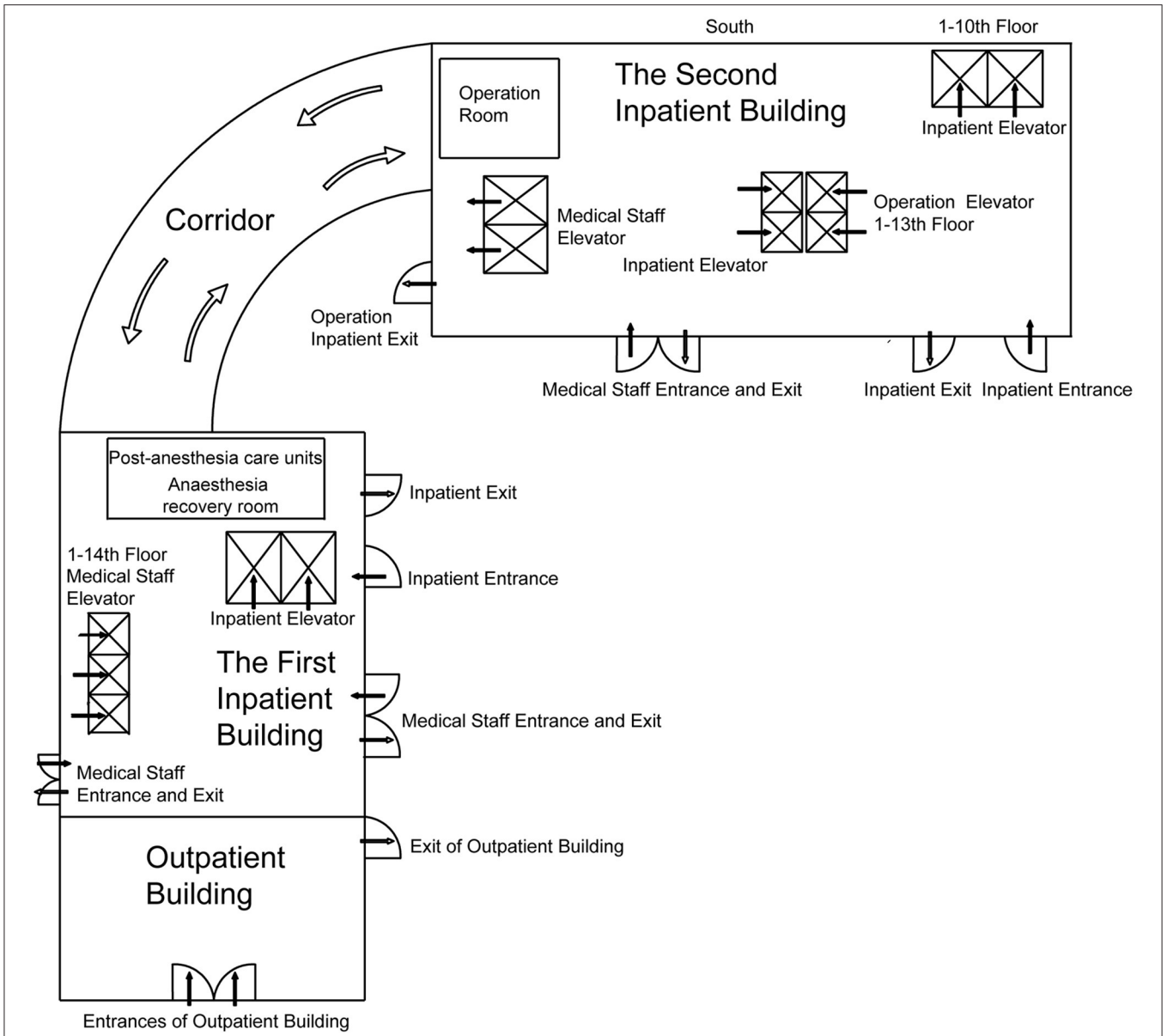


FIGURE 2 | The building plan of the outpatient building and inpatient buildings 1 and 2. The post-anesthesia care units are on the 10th floor of the first inpatient building, and the operating rooms are on the 11th to 13th floor of the second inpatient building. The two buildings are connected by corridors.

5. The use of antiemetics could lessen postoperative nausea, vomiting, and the possibility of exposure.
6. When patients with tracheotomy needed sputum suction, medical staff had to wear anti-seepage isolation clothing and goggles or a face screen on the basis of standard prevention (5).
7. After the patient left the anesthesia recovery room, the used instruments, surface, bed unit, cardiac conductivity line, blood pressure cuff, blood oxygen saturation fingertip, etc. were wiped with a disinfectant containing 500 mg/L chlorine, and the humidification bottle was placed into a white garbage bag with a cover.
8. When transferring patients back to the ward, the shortest route and the special elevator for surgical patients were taken.
9. The disposable bedspread and quilt cover were replaced by one person only, and the part of the transfer vehicle that had come into contact with the patient was wiped with a disinfectant containing 500 mg/L chlorine.

Special Management of Suspected/Confirmed Patients

In theory, suspected/confirmed patients should have no elective surgery, as the impact of surgery may aggravate the patient's condition or even lead to the death of the patient. Only

emergency rescue surgery was performed (6). The operation and postoperative recovery were completed in a negative-pressure operating room.

Aside from the routine recovery nursing, the following should be paid attention to when patients are being intubated and recovering in the negative-pressure operating room (7):

1. The air pollution in the operating room should be lessened as much as possible, a breathing filter at the connection between the patient's tracheal intubation and the threaded pipe of the anesthesia machine should be installed, and a breathing filter between the suction and the exhalation of the anesthesia machine should be installed as well.
2. Under deep anesthesia, secretion in the airway should be suctioned to lessen the incidence of cough.
3. There should be someone to assist the anesthesiologist in the removal of the tracheal intubation when the patient's spontaneous breathing pattern has not recovered.
4. When the tracheal tube is pulled out, the breathing filter at the end of the tube should be kept and connected with the breathing mask, which should be tightly connected with the patient's nose and mouth and connected with the anesthetic breathing circuit.
5. Following the stoppage of oxygen inhalation, the patient should wear a medical protective mask right away.
6. The prophylactic use of antiemetic drugs to avoid nausea and vomiting complications should be noted.
7. Once the patient is deemed ready for discharge, the infection management department and the medical department of the hospital should be asked to work out the transfer route and make preparations for the transfer route and docking with the isolation ward.
8. The transport personnel ought to wear protective equipment in accordance with the level three protection standard, carry a sealed special transport rescue box (including the breathing bag, breathing mask, sputum suction tube, and 50-ml syringe), and use a negative-pressure transfer bed to transport the patients back to the isolation ward.
9. The treatment of articles, instruments, and equipment used by patients and the environmental treatment of the operating room and buffer room should be performed in accordance with the regulations on the management of medical wastes, the technical specifications for disinfection of medical institutions, the management specifications for hospital air purification, and the management specifications for environmental surface cleaning and disinfection of medical institutions.
10. The medical staff in the operating room should only leave the negative-pressure operating room after taking off their medical protective equipment in accordance with the standard process and hand hygiene.

TRAINING MANAGEMENT OF MEDICAL PERSONNEL

Training on the Front Line

The hospital adopted the forms of live broadcast, a TV morning meeting, WeChat enterprise number push, and so on, to report COVID-19-related knowledge to medical staff, students, and logistics personnel in all hospitals and to train the personnel on how to wear protective equipment and relieve mental stress (8). At the end of each course, an online theoretical assessment was performed to complete the first step of advanced training.

Offline Training After Arrival

The selection of personal protective equipment (PPE) in place of standard and corresponding protective measures and various treatment activities in clean areas, potential pollution areas, and pollution areas was made clear. The aim was that protection of medical personnel would be implemented, but overprotection would be ended. When nurses assist in tracheal intubation, sputum suction, tracheal intubation extubation, fiberoptic bronchoscopy, etc., which may generate splashes or aerosols, they should wear medical protective masks, goggles/protective screens, and anti-seepage isolation clothing (9). It was highlighted that the corresponding protective equipment should be removed in accordance with the standard process when leaving the polluted area and potentially polluted areas, and, as far as possible, pollution should not happen during the removal. Since the wearing of protective equipment impacts the operation sensitivity and flexibility of nursing staff, the wearing of three-level protective equipment will also impact the hearing, vision, and touch of nursing staff, resulting in poor communication between colleagues, poor operations, and even operation failure (10, 11). Thus, situation simulation training of wearing protective equipment should be performed in batches to lessen the negative impact of wearing protective equipment on nurses (**Figure S1**).

We outline recommendations for the PPE of the medical staff in anesthesia surgery centers for three-level screening (**Table 1**).

For patients with no fever during selective operation, we carried out level 1 protection (**Figure 3A**): Work clothes, disposable work caps, and disposable surgical masks were to be worn. Latex gloves were also worn when in contact with blood, body fluids, secretions, or excreta, and goggles or a protective face screen were worn when carrying out tracheal intubation, sputum aspiration, tracheal extubation, and other possible ways to generate aerosols. For the replacement time, disposable surgical masks were to be changed every 4 h if they were not contaminated or wet. Disposable working caps and clothes had to be changed every 8 h and replaced in time if contaminated or wet; the goggles or protective screen had to be replaced following each operation.

For patients with fever during selective operation, we implemented level 2 protection (**Figure 3B**): Work clothes, disposable work caps, and disposable medical protective masks (N95 type masks and above), disposable protective clothing, disposable waterproof isolation clothes, waterproof shoe covers, double latex gloves, and goggles or a protective face screen were worn. In terms of replacement time, disposable medical protective masks had to be replaced every 4 h if not contaminated or wet. Disposable working caps and clothes had to be replaced every 8 h and were replaced in time if contaminated or wet; following each operation, the disposable waterproof

TABLE 1 | Recommendations for the personal protective equipment (PPE) of the medical staff in anesthesia surgery centers.

Protective level	Scope of application	PPE	Replacement time
Level 1	Patients with no fever during elective operation	Work clothes, disposable work caps, and disposable surgical masks were to be worn. Latex gloves were also worn when in contact with blood, body fluids, secretions, or excreta, and goggles or protective face screen were worn when carrying out tracheal intubation, sputum aspiration, tracheal extubation, and other possible ways to generate aerosols.	Disposable surgical masks had to be changed every 4 h if they were not contaminated or wet. Disposable working caps and clothes had to be changed every 8 h and were to be replaced in time if contaminated or wet; goggles or protective screens had to be replaced following each operation.
Level 2	Patients with fever during elective operation	Work clothes, disposable work caps and disposable medical protective masks (N95 type masks and above), disposable protective clothing, disposable waterproof isolation clothes, waterproof shoe covers, double latex gloves, and goggles or protective face screens were worn.	Disposable medical protective masks had to be replaced every 4 h if not contaminated or wet. Disposable working caps and clothes had to be replaced every 8 h and had to be replaced in time if contaminated or wet; following each operation, the disposable waterproof protective clothing, disposable protective clothing, waterproof shoe covers, and goggles or protective face screen were to be replaced.
Level 3	Patients with suspected or confirmed COVID-19 during emergency surgery	Work clothes, disposable working cap and comprehensive respirator, disposable protective clothing, disposable waterproof isolation clothes, waterproof shoe covers, and double latex gloves were worn.	All equipment had to be replaced after each operation.

protective clothing, disposable protective clothing, waterproof shoe cover, and goggles or protective face screen had to be replaced.

For patients with suspected or confirmed COVID-19 during emergency surgery, we carried out level 3 protection (**Figure 3C**): work clothes, disposable working cap, and comprehensive respirator, disposable protective clothing, disposable waterproof isolation clothes, waterproof shoe covers, and double latex gloves were worn. In terms of the replacement time, all equipment was replaced following each operation.

SPECIAL ENVIRONMENTAL AND HUMAN RESOURCE MANAGEMENT

Strict Environmental Management

Windows were opened to ventilate, the air conditioning was turned off, and the air was disinfected thrice a day in the anesthesia recovery room, the office area, and the dining room. Office desktops, mice, keyboards, printers, walkie talkies, computers, and other public facilities had to be wiped with 500 mg/L chlorine-containing disinfectant thrice a day. The door handles of public toilets, changing rooms, and duty rooms had to be disinfected every day, and toilet paper was provided outside the door for use as an anti-pollution measure. To avoid cluster-dining and cross-infection, the dining plan of the dining room of the anesthesia operation center was adjusted: self-service dining was canceled and modified to aid with taking a box meal; the dining time was controlled to about 20 min in batches and to a limited number of people, and no chatting was allowed during the dining period; most of the dining chairs were removed to ensure that the distance between each diner was not < 1 m.

Human Resource Management Under Special Situations

We listed details on working shift durations for various employees. Recently, research has indicated that we should consider minimizing staff exposure to COVID-19 patients by optimizing work shifts (12). The numbers of open operating rooms, anesthesiologists, and anesthesia nurses who needed to work were determined in accordance with the amount of surgery on the next day. The working time of medical staff was around 8–10 h with rest breaks. The anesthesiologists in the surgery room were divided into three batches with three different shifts, 8:00–16:00, 9:00–17:00, and 16:00–19:00; the anesthesiologists in PACU were split into two batches with three shifts, 7:00–17:30 and 9:00–19:00; the nurses and transport workers were split into four batches with four shifts: 8:00–16:00, 9:00–17:00, 10:00–18:00, and 11:00–19:00. These schedules ensured the smooth operation and lessened staff gathering. Cleaning personnel were split into two batches with two shifts, 7:00–15:00 and 15:00–23:00, for two-liner change.

ONLINE TEACHING MANAGEMENT

Our hospital is the teaching hospital of a large medical center. Because of the impact of the epidemic situation and the delay of the students' school opening time, we investigated new teaching methods and conducted live broadcasts, question answering, and discussions of theoretical courses via various networked teaching or conferencing platforms. To avoid the gathering of personnel, on-site teaching was canceled and modified to record teaching videos for the Wechat group, asking and answering questions from the group. After 3 days of file sharing, online tests related to the teaching content were taken to test the learning effect and ensure the teaching quality. During

FIGURE 3 | Personal protective equipment for medical staff at three levels. **(A)** Level 1 protection: for patients with no fever during elective operation. **(B)** Level 2 protection: for patients with fever during elective operation (wear disposable medical latex gloves with an outer layer). **(C)** Level 3 protection: for patients with suspected or confirmed COVID-19 during emergency surgery (wear disposable medical latex gloves with an outer layer).

the COVID-19 epidemic, we should strengthen the training of interns on the knowledge and skills related to epidemic prevention and control and pay special attention to training on the awareness, skills, and psychological adjustment of prevention and control. During the outbreak of COVID-19, interns did not carry out invasive operations so as to lessen the possibility of occupational exposure.

DISCUSSION

Under the COVID-19 pandemic, the ways to ensure the gradual recovery of anesthesia nursing unit and avoid cross-infection of the anesthesia nursing unit in a West China hospital can be outlined according to six aspects.

1. Hospital and operating room channel management: The hospital and the nursing department should adopt a reasonable layout of the medical space, optimize the treatment process and patient transfer process, implement "three-channel management," and build a physical barrier.
2. Three-level screening management of patients and medical staff: Patients and medical staff should perform epidemiological history screening and be under the control of three-level screening management to implement multiple filtering and cut off the source of infection.
3. Classification management of patients undergoing anesthesia and recovery: Ordinary patients and suspected/confirmed patients with COVID-19 should be managed in accordance with anesthesia and operation classification and precautions to ensure the safety of patients and staff.
4. Training management of medical personnel: Training should be given to medical staff on COVID-19 prevention and control to improve personal protection ability, particularly covering medical staff nursing behavior, selection of protective equipment, and specification of the wearing and taking off process based on "three-level protection" under different situations.
5. Special environmental and human resource management: The strict management of the environment of the department should be strengthened, aggregation lessened, and the supply of ppe ensured; flexible human resource management can ensure the smooth completion of daily work while reducing the number of medical staff and exposure as much as possible;
6. Online teaching management: On-site teaching should be replaced with online teaching to ensure the safety of students and to complete the teaching plan.

Some other groups have also shared their clinical experiences of managing patients under COVID-19. Sorbello et al. (13) described key elements of clinical management in Italy, including safe oxygen therapy, airway management, PPE, and non-technical aspects of caring for patients diagnosed with COVID 2019. In these settings, there are specific factors that must be highlighted: oxygen administration and non-invasive ventilation of spontaneously ventilating patients; airway management of patients requiring tracheal intubation; clinical management with PPE; and human factors. Dexter et al. (14) suggested

an evidence-based approach for the optimization of infection control and operating room management to defend perioperative COVID-19. The approach included improved hand hygiene, environmental cleaning via surface disinfectants and ultraviolet light, improved vascular care, patient decolonization, and surveillance optimization, which was in part consistent with our strategies. Recently research indicated that a combination of effective patient testing strategies, intelligent work planning, and thoughtful resource management could optimize treatment capacity, limit healthcare worker exposure, limit unnecessary use of PPE, and ensure high-quality patient care while avoiding staff overexertion (12, 15).

Through the implementation of the previously mentioned epidemic prevention and control strategies, anesthesia nursing work in our department is performed in an orderly and safe manner. Theoretical teaching is arranged according to the plan, but online teaching and discussion are more popular with students. These epidemic prevention and control strategies are based on China's national conditions, local epidemic situation, and hospital conditions, so anesthesia nursing colleagues can select from them in accordance with their own specific conditions.

AUTHOR CONTRIBUTIONS

PZ, RZ, LYe, and TZ contributed to the conception and design of the study. LYi, XY, YM, HW, LYe, and TZ contributed to the acquisition, analysis, and interpretation of data. All authors were involved in the revision of the manuscript, provided intellectual content of critical importance, and read and gave final approval of the version to be published.

ACKNOWLEDGMENTS

We would like to acknowledge colleagues at the Anesthesiology/Nursing Department of West China Hospital combating COVID-19.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2020.00460/full#supplementary-material

Figure S1 | Training on protective equipment for medical staff in the operating room. **(A)** Level 1 protection training; **(B)** level 2 protection training.

REFERENCES

1. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet.* (2020) 395:497–506. doi: 10.1016/S0140-6736203 0183-5
2. Zhu N, Zhang D, Wang W, Li X. A novel coronavirus from patients with pneumonia in China, 2019. *N Engl J Med.* (2020) 382:727–33. doi: 10.1056/NEJMoa2001017
3. Han Y, Yang H. The transmission and diagnosis of 2019 novel coronavirus infection disease (COVID-19): a Chinese perspective. *J Med Virol.* (2020) 92:639–44. doi: 10.1002/jmv.25749
4. Li Y, Xia L. Coronavirus disease 2019 (COVID-19): role of chest CT in diagnosis and management. *AJR Am J Roentgenol.* (2020) 214:1280–6. doi: 10.2214/AJR.20.22954
5. Luo M, Cao S, Wei L, Tang R, Hong S, Liu R, et al. Precautions for intubating patients with COVID-19. *Anesthesiology.* (2020) 132:1616–8. doi: 10.1097/ALN.0000000000003288
6. Zhang HF, Bo LL, Lin Y, Li FX, Sun SJ, Lin HB, et al. Response of Chinese anesthesiologists to the COVID-19 outbreak. *Anesthesiology.* (2020) 132:1333–8. doi: 10.1097/ALN.0000000000003300
7. Chen X, Liu Y, Gong Y, Guo X, Zuo M, Li J, et al. Perioperative management of patients infected with the novel coronavirus: recommendation from the joint task force of the Chinese society of anesthesiology and the Chinese association of anesthesiologists. *Anesthesiology.* (2020) 132:1307–16. doi: 10.1097/ALN.0000000000003301
8. Li Z, Ge J, Yang M, Feng J, Qiao M, Jiang R, et al. Vicarious traumatization in the general public, members, and non-members of medical teams aiding in COVID-19 control. *Brain Behav Immun.* (2020). doi: 10.1016/j.bbi.2020.03.007. [Epub ahead of print].
9. Meng L, Qiu H, Wan L, Ai Y, Xue Z, Guo Q, et al. Intubation and ventilation amid the COVID-19 outbreak: Wuhan's experience. *Anesthesiology.* (2020) 132:1317–32. doi: 10.1097/ALN.0000000000003296
10. Tien HC, Chughtai T, Jogeklar A, Cooper AB, Brenneman F. Elective and emergency surgery in patients with severe acute respiratory syndrome (SARS). *Can J Surg.* (2005) 48:71–4.
11. Wong J, Goh QY, Tan Z, Lie SA, Tay YC, Ng SY, et al. Preparing for a COVID-19 pandemic: a review of operating room outbreak response measures in a large tertiary hospital in Singapore. *Can J Anesthe.* (2020) 67:732–45. doi: 10.1007/s12630-020-01620-9
12. Mascha EJ, Schober P, Schefold JC, Stueber F, Luedi MM. Staffing with disease-based epidemiologic indices may reduce shortage of intensive care unit staff during the COVID-19 pandemic. *Anesth Analg.* (2020) 131:24–30. doi: 10.1213/ane.0000000000004849
13. Sorbello M, El-Boghdadly K, Di Giacinto I, Cataldo R, Esposito C, Falcetta S, et al. The Italian coronavirus disease 2019 outbreak: recommendations from clinical practice. (2020) 75:724–32. doi: 10.1111/anae.15049
14. Dexter F, Parra MC, Brown JR, Loftus RW. Perioperative COVID-19 defense: an evidence-based approach for optimization of infection control and operating room management. *Anesth Analg.* (2020) 131:37–42. doi: 10.1213/ane.0000000000004829
15. Holthof N. Preparing for the aftermath of COVID-19: important considerations for healthcare providers and hospital administrators. *Anesth Analg.* (2020) 131:pe50–e51. doi: 10.1213/ane.0000000000004911

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 22 July 2020
doi: 10.3389/fmicb.2020.01800

Geographic and Genomic Distribution of SARS-CoV-2 Mutations

Daniele Mercatelli† *and Federico M. Giorgi**†

Department of Pharmacy and Biotechnology, University of Bologna, Bologna, Italy

The novel respiratory disease COVID-19 has reached the status of worldwide pandemic and large efforts are currently being undertaken in molecularly characterizing the virus causing it, SARS-CoV-2. The genomic variability of SARS-CoV-2 specimens scattered across the globe can underly geographically specific etiological effects. In the present study, we gather the 48,635 SARS-CoV-2 complete genomes currently available thanks to the collection endeavor of the GISAID consortium and thousands of contributing laboratories. We analyzed and annotated all SARS-CoV-2 mutations compared with the reference Wuhan genome NC_045512.2, observing an average of 7.23 mutations per sample. Our analysis shows the prevalence of single nucleotide transitions as the major mutational type across the world. There exist at least three clades characterized by geographic and genomic specificity. In particular, clade G, prevalent in Europe, carries a D614G mutation in the Spike protein, which is responsible for the initial interaction of the virus with the host human cell. Our analysis may facilitate custom-designed antiviral strategies based on the molecular specificities of SARS-CoV-2 in different patients and geographical locations.

Keywords: SARS-CoV-2, genome evolution, COVID-19, genomics, coronavirus

OPEN ACCESS

Edited by:
Anna Kramvis,
University of the Witwatersrand, South Africa

Reviewed by:
Longping Victor Tse,
The University of North Carolina at Chapel Hill, United States
Tetsuro Ikegami,
The University of Texas Medical Branch at Galveston, United States

****Correspondence:***
Federico M. Giorgi
federico.giorgi@unibo.it

†ORCID:
Daniele Mercatelli
orcid.org/0000-0003-3228-0580
Federico M. Giorgi
orcid.org/0000-0002-7325-9908

Specialty section:
This article was submitted to Virology, a section of the journal Frontiers in Microbiology

Received: *27 April 2020*
Accepted: *09 July 2020*
Published: *22 July 2020*

Citation:
Mercatelli D and Giorgi FM (2020) Geographic and Genomic Distribution of SARS-CoV-2 Mutations. Front. Microbiol. 11:1800. doi: 10.3389/fmicb.2020.01800

INTRODUCTION

Initially reported in mid-December 2019 in the Chinese city of Wuhan, the newly emerged severe acute respiratory syndrome virus (SARS-CoV-2) is a single-stranded RNA beta-coronavirus with a compact 29,903 nucleotides-long genome. This virus causes a serious disease known as Coronavirus Disease 2019 (COVID-19), which has spread in over 210 countries in <4 months, counting more than 10 million confirmed cases and almost 500,000 deaths reported worldwide as of June 28, 2020 (source: World Health Organization). A difference in case fatality rates across countries was observed, possibly due to a diverse demographic composition and the type of measures that have been taken in different countries to limit viral spreading (Dowd et al., 2020). According to data from the public database of the Global Initiative on Sharing All Influenza Data (GISAID), three major clades of SARS-CoV-2 can be identified (Forster et al., 2020), that have been subsequently named as clade G (variant of the spike protein S-D614G), clade V (variant of the ORF3a coding protein NS3-G251), and clade S (variant ORF8-L84S). However, as more complete sequences become available, the need to define specific geographic distributions of virus variants becomes of practical importance to define clinical and political strategies at the local level. Despite several reports having confirmed a relatively low variability of SARS-CoV-2 genomes (Ceraolo and Giorgi, 2020; Lu et al., 2020), it is still unclear if different fatality rates or speed of transmission observed

in different countries may be the consequence of clade's differences in virulence, as discussed by a recent commentary comparing different strains in the USA (Brufsky, 2020). It is therefore possible that more insights into the pathogenesis and virulence of this virus may come from comparative genomic analysis linked to epidemiologic data coming from different countries.

Genetic variance analyses must now play a crucial role in expanding knowledge on this new virus to adopt measures to contain its outbreak. Complete viral genome sequences have been made rapidly publicly available to the research community and have recently surpassed the 48,000 units, thanks to the worldwide effort of scientists and to the GISAID consortium. This data avalanche will result in an unprecedently rapid effort to analyze data to understand genome diversity (Andersen et al., 2020; Shen et al., 2020), to hypothesize suitable targets for drug repositioning (Wu et al., 2020; Zhou et al., 2020) and to develop prevention strategies (Zhao and Chen, 2020). In the present study, we performed the largest comparative study so far by analyzing more than 48,000 complete SARS-CoV-2 genomes. We report all mutations and stratify them genomically and geographically, also highlighting insurgence of sub-clades and genomic highly variable spots. These finding may be extremely useful to design and think about the efficacy of measures that have been taken on a regional basis to limit SARS-CoV-2 spreading.

METHODS

Forty-eight thousand six hundred thirty-five SARS-CoV-2 genomic sequences were downloaded from GISAID (Shu and McCauley, 2017) on June 26, 2020 (**Supplementary File 1**). Only viruses affecting human hosts were selected, removing low-quality sequences (>5% NNNs) and using only full-length sequences (>29,000 nt). Forty-eight thousand six hundred twenty-four sequences were associated to a geographic region, specifically: 514 from Africa, 3,340 from Asia, 31,818 from Europe, 10,250 from North America, 2,127 from Oceania and 575 from South America. Eleven sequences were not associated to any continent. We provide as **Supplementary File 2** a full geographic description of each sample used in the study.

The reference NC_045512.2 SARS-CoV-2 Wuhan genome (Coronaviridae Study Group of the International Committee on Taxonomy of Viruses, 2020), 29,903 nucleotides long, was obtained from NCBI GenBank. A GFF3 annotation associated to the refence, showing genomic coordinates for all protein sequences of SARS-CoV-2, is provided as **Supplementary File 3**. The large ORF1 polyprotein was split into its constituent Non-structural proteins (NSPs). The NSP12, encoding for the viral RNA-dependent RNA polymerase, was considered in the annotation as two regions, NSP12a and NSP12b, corresponding to the regions before and after a ribosomal frameshift, occurring as nucleotide 13,468 is translated as both the last nucleotide of a codon and the first of the next codon.

NUCMER version 3.1 (Delcher, 2002) was used to align all 48,635 genome sequences over the NC_045512.2 reference. The output of the alignment was converted to an annotated list of all mutational events using an internally developed R SARS-CoV-2 annotation algorithm provided as **Supplementary File 4**.

SARS-CoV-2 5′UTR RNA secondary structure has been predicted by free energy minimization together with equilibrium partition function and base pair binding probabilities algorithm from the RNAfold WebServer using default settings (Gruber et al., 2008).

RESULTS

Our analysis of 48,635 SARS-CoV-2 highlights a total of 353,341 mutation events compared to the NC_045512.2 Wuhan reference genome. Our results, event by event, are available as **Supplementary File 5**. While 256 samples, mostly originating from Asia, did not have any difference from the reference, 48,379 samples possessed at least one mutation. The number of mutations is relatively low, with mode per sample equaling 6, an average of 7.23, and very few samples having more than 15 events (**Figure 1A**). Overall, no continent differs significantly from the average mutation rate (**Figure 1B**), but there is a significant difference (one-way ANOVA $p = 9.55 \times 10^{-205}$) in the average number of mutations per sample between countries. Specifically, amongst the top 40 nations with the highest number of sequenced full viral genomes (**Figure 1C**), these countries have a slightly but significant higher number of observed mutations per sample, when compared to the world's average: India: (8.40), Congo (8.30), Bangladesh (9.83), and Kazakhstan (9.47). On the other hand, the sequences from the following countries show a significantly lower mutational burden: Germany (6.09), Japan (4.55), Italy (5.92), Greece (5.91), Hong Kong (5.00), and Kenya (5.38). One must bear in mind that some sampling biases may affect this comparison: for example, some countries have generated the highest number of sequences in the early phases of the pandemic, and may have therefore observed fewer mutations (for example, Italy has not shared any sequence in the months of May and June 2020, the last two considered in our analysis). On the other hand, one would expect China to have a lower number of mutations, being the likely point of origin of SARS-CoV-2 (Ceraolo and Giorgi, 2020), and indeed the distribution of mutations per sample seems to suggest that (**Figure 1C**); however, a small number of sequences carrying a very high number (>50) of mutations are associated to China, shifting the distribution for this country. Upon manual inspection, these sequences do not appear to share similarities between each other, and are likely the product of technical sequencing errors.

We analyzed the nature of each mutation, highlighting a massive prevalence of single-nucleotide polymorphisms (SNPs) over short insertion/deletion events (indels) worldwide (**Supplementary File 6**) and in every continent (**Figure 2A**). Worldwide, we observed 205,482 amino acid(aa)-changing SNP events (58.2% of the total), with fewer than half silent SNPs falling

Abbreviations: AA, amino acid; COVID-19, Coronavirus Disease 2019; GISAID, Global Initiative on Sharing All Influenza Data; Indel, insertion/deletion event; NSP, non-structural protein; ORF, open reading frame; S, SARS-CoV-2 spike protein; SARS-CoV-2, Severe Acute Respiratory Syndrome, Coronavirus 2; SNP, single nucleotide polymorphism.

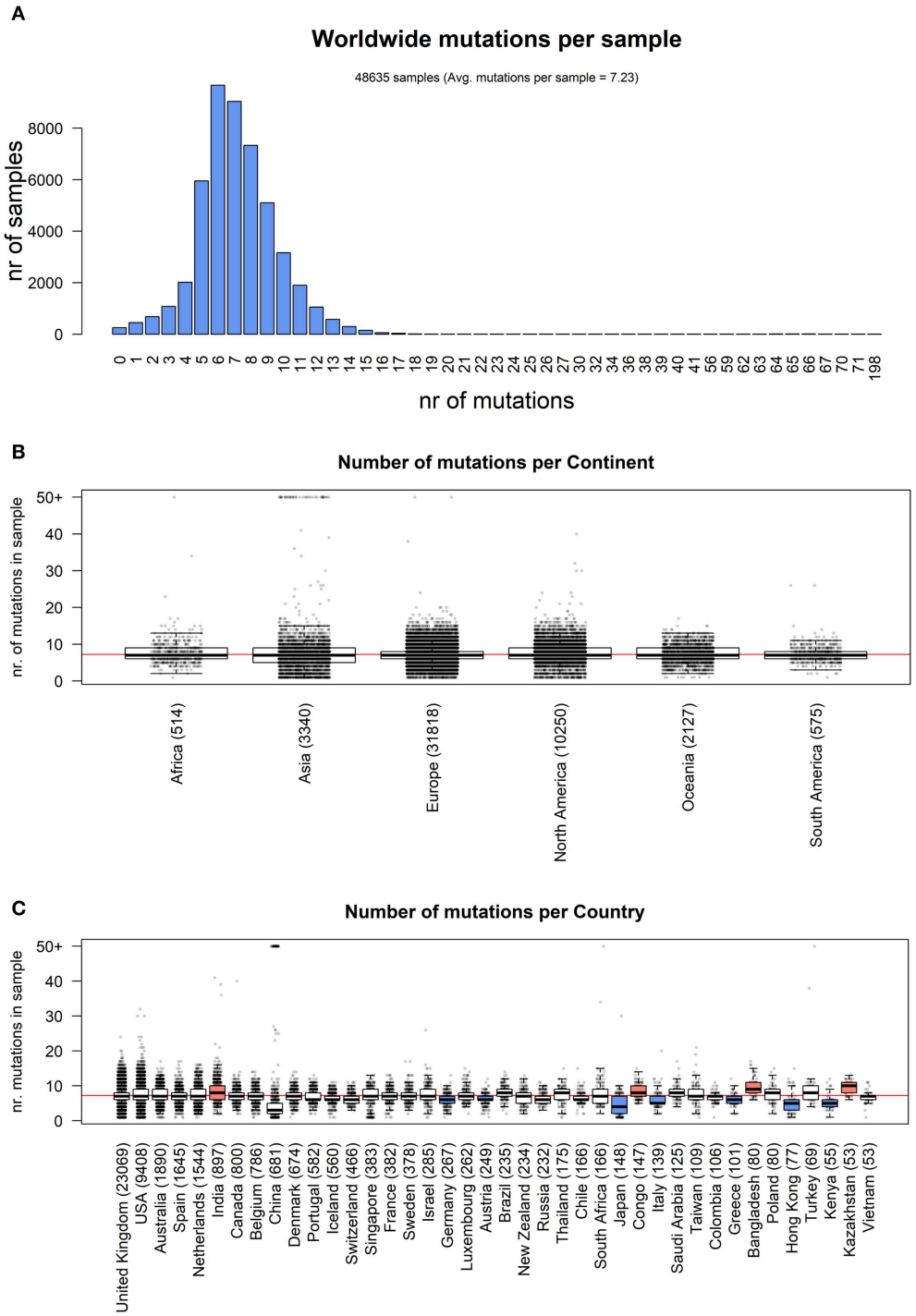


FIGURE 1 | (A) Distribution of number of mutational events for all SARS-CoV-2 genome samples analyzed. **(B)** Distributions of number of mutations for each sample, stratified per continent. The main boxplot rectangles are drawn between the 1st and 3rd quartile, with the median value indicated as a thick line. Boxplot whiskers fall on the closest point to the 1st/3rd quartile + 1.5 interquartile range as described in the R boxplot() function. The number in brackets after the continent name indicates the number of sequenced genomes. The horizontal red line indicates the average number of mutations per sample, worldwide. **(C)** As in **(B)**, with stratification performed country-wise, using the 40 countries with the highest number of sequenced genomes. The boxplot color indicates the country has a mutation rate higher (red) or lower (blue) than the world's average (Kolmogorov-Smirnov test $p < 2.2 \times 10^{-16}$ and absolute difference of averages between country and world higher than one).

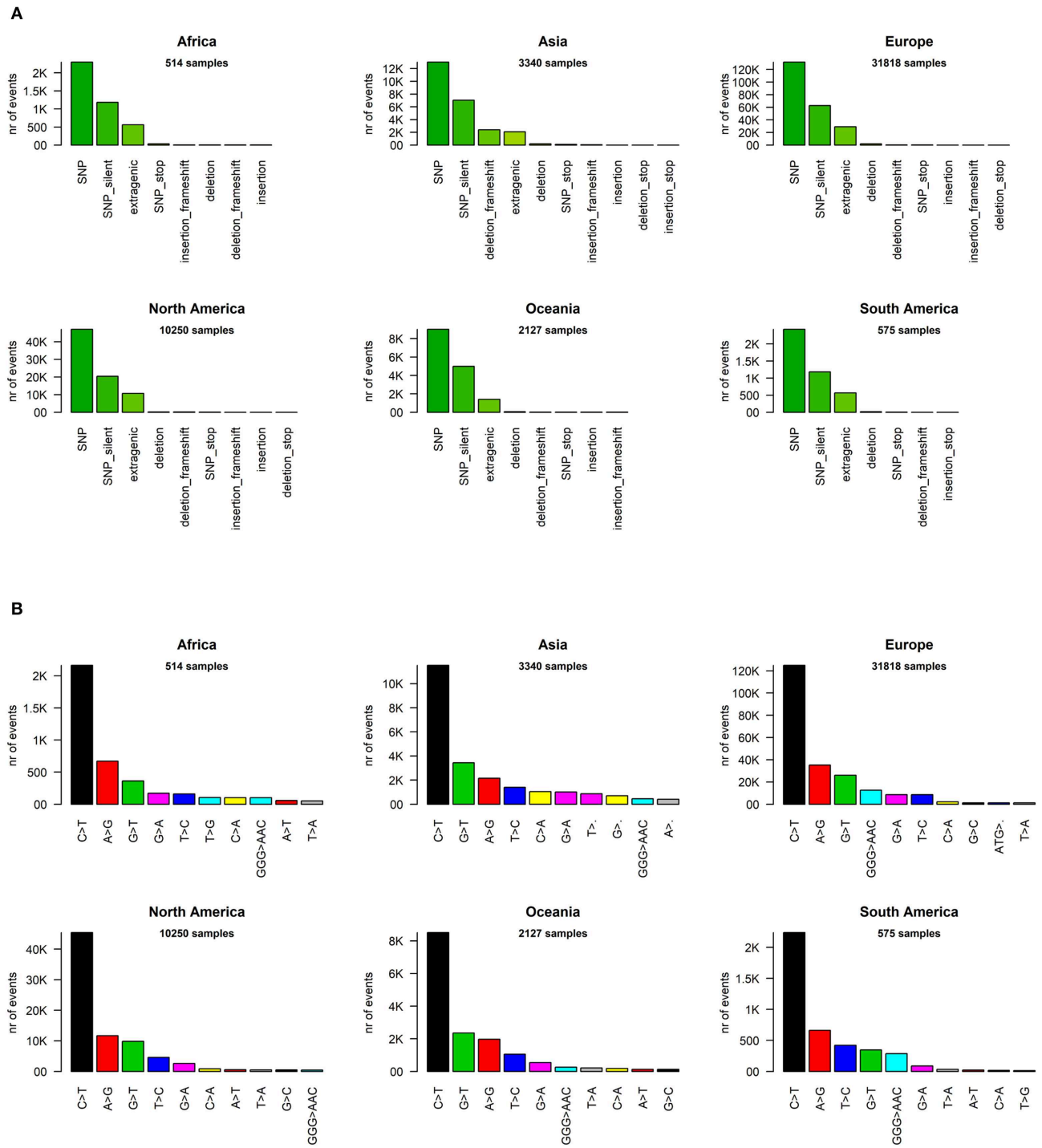


FIGURE 2 | (A) Distribution of SARS-CoV-2 mutation classes in continents. "SNP," "deletion," and "insertion" terms without further specifications are intended as frameshift-preserving aa-changing events. **(B)** Continent-stratified distribution of SARS-CoV-2 mutation types. Colors are assigned randomly but preserved across panels to facilitate tracking of identical types across continents. Listed nucleotide changes represent those found in the positive-sense viral RNA. We indicate the thymine T letter for consistency with the NCBI reference sequence NC_045512.2, but the actual viral sequence will be factually represented by a U (uracil) as the RNA counterpart for thymine. Dots ('·') on the x-axis mutation type names indicate nucleotide deletion.

in coding regions (27.6%, with 97,573 events). There are 44,345 events in intergenic regions (12.6%), prevalently the 5′UTR and 3′UTR of the SARS-CoV-2 RNA sequence. Short frameshift deletions are the most common indel event in the SARS-CoV-2 population (0.8%), followed by in-frame deletions (3x deletions reducing the viral protein length without introducing

stop codons), which account for 0.6% of all observed mutational events. SNPs generating a stop codon are also very rare (496 observed events, 0.1% of the total). Insertions are an extremely rare event, accounting for <0.1% of all SARS-CoV-2 mutations detected so far. Similar profiles and relative percentages are observed in all continents, suggesting a conserved molecular basis for SARS-CoV-2 evolution (**Figure 2A**).

We then classified the SARS-CoV-2 mutations according to their type, observing a prevalence of SNP transitions (purine->purine and pyrimidine->pyrimidine) over SNP transversions (purine->pyrimidine and vice versa), an observation that matches what was observed for SARS-CoV (Hu et al., 2003). The most common event, both worldwide and continent-wise, is by far the C>T transition, accounting for 55.1% of all observed worldwide viral mutations (**Figure 2B**, **Supplementary File 6**). The A>G transition is the second most common event worldwide (14.8%) and in Africa, Europe, and the Americas. The most common transversion, G>T, is the third most common event worldwide, with 42,408 occurrences (12.0%), but it is the second most common event in Asia and Oceania. The most common indel, the deletion of the ATG codon, is the 12th most common event worldwide, with a total of 1,298 occurrences, but it rises to the 9th most frequent in European genomes (**Figure 2B**). A peculiar multi-nucleotide event, the substitution of a GGG triplet with AAC, was also observed as the 5th most common event worldwide (4.0%, **Supplementary File 6**). As we will discuss later, this mutation type is mostly associated to a specific event affecting the Nucleocapsid locus, which characterizes the clade GR in the viral phylogenetic tree. It must be noted here that our choice of the "T" base notation, corresponding to thymine, was made for compatibility reasons with the NCBI NC_045512.2 reference genome notation, while the actual RNA base in the SARS-CoV-2 genome is a "U" (Uracil).

We went into higher detail and analyzed the effects of each mutation on the protein sequences of SARS-CoV-2. Again, the profiles appear quite similar across continents. The most prevalent mutation in sequenced genomes worldwide is a transversion affecting the 23,403rd nucleotide adenosine (**Supplementary File 6**), transformed into a guanosine (A23403G), defining the so-called G-clade of SARS-CoV-2 genomes, prevalent in Europe (where overall the highest sequencing effort has been undertaken, and therefore the highest number of samples), Oceania, South America, and Africa (**Figure 3A**). This mutation causes a D614G (aspartate to glycine in protein position 614) aa-change of the Spike (S) protein, which is responsible for the initial entry of the virus in the cell via the ACE2 human receptor (Guzzi et al., 2020). However, this mutation is outside the observed Spike/ACE2 binding domain, roughly located between amino acids 330 and 530 (Wang et al., 2020). Three mutations show similar frequency with A23403G: C14408T, C241T, and C3037T (**Figure 3A**). As we will show later, these four mutations are almost always co-occurring in the same genomes, defining the major clade G observed in the viral population. In Asia, while the most common mutation was G11083T for samples sequenced between December 2019 and March 2020, recent sequencing efforts have highlighted a current profile similar to those of the other continents (**Figure 3A**).

The effect of the majority of SARS-CoV-2 nucleotide mutations is reflected in protein changes. We show, in **Figure 3B**, the most common mutations, in protein annotation, in the six continents, while in **Table 1** we highlight the effect of the 20 most common mutations worldwide, in nucleotide and protein coordinates. The most common set of events is a quadruplet of mutations, corresponding to the G clade nucleotide mutations described before. Apart from the aforementioned D614G mutation observed in the Spike protein, the second most common amino acid changing mutation is P314L, affecting the Non-structural Protein 12 (NSP12), the viral RNA-dependent RNA polymerase. The other two mutations in the top four do not affect the protein sequence, as they are silent mutations targeting the 106th codon of NSP3 (a viral predicted phosphoesterase) and the 5′UTR in position 241.

Other common mutations affecting protein sequence are N:RG203KR (in the Nucleocapsid protein N), induced by a tri-nucleotide mutation and determining a 2-amino acid change and mutations affecting the less characterized ORF3a, ORF8, NSP2, NSP6, and NSP13 proteins (**Table 1**). The G15S mutation in the viral protease NSP5 is the 16th most common event worldwide, with 1,798 samples affected (3.7%), however it seems to be too peripheric, in the protein sequence, to influence catalytic activity, and folding (Zhang et al., 2020).

We proceeded then to analyze the distribution of mutation groups rather than individual events, in order to observe their phylogenetic groups and geographical and temporal distributions. Our observation on co-occurring mutations (**Figure 4**) matches the current phylogenetic classification defined by the GISAID consortium (**Table 2**). Specifically, the four mutations C241T, C3037T, C14408T, and A23403G are observed in all samples from the clade "G" (named after the Spike D614G mutation) and its two derivative GH (further characterized by the ORF3a:Q57H mutation) and GR (affected by the trinucleotide mutation in the Nucleocapsid gene, inducing a RG203KR mutation).

Other two major clades are called "S," named after the mutation in ORF8 L84S (Ceraolo and Giorgi, 2020), also characterized by a silent C8782T genomic mutation, and "V," from the ORF3a:G251V mutation, almost always co-occurring with the NSP6:L37F event, and identified by early phylogenetic studies (Forster et al., 2020). The original lineage "L," corresponding to the reference genome NC_045512.2, is populated in our study by all genomes carrying reference alleles for all loci defined in clades G, GH, GR, S, and V (**Table 2**). Finally, a general group for other sequences not matching any of these criteria (e.g., other alleles or combinations) is defined here as "O" clade. Clustering all genomes clearly highlights the five major phylogenetic groups G, GH, GR, S, and V and their characterizing mutations (**Figure 4**), as well as more nascent clades (e.g., in the GH clade, further split by a novel mutation in the NSP2 locus, C1059T), and a general distribution of non-recurring mutations for the majority of sequences. There are, however, a few hundreds of highly "clean" sequences (e.g., for

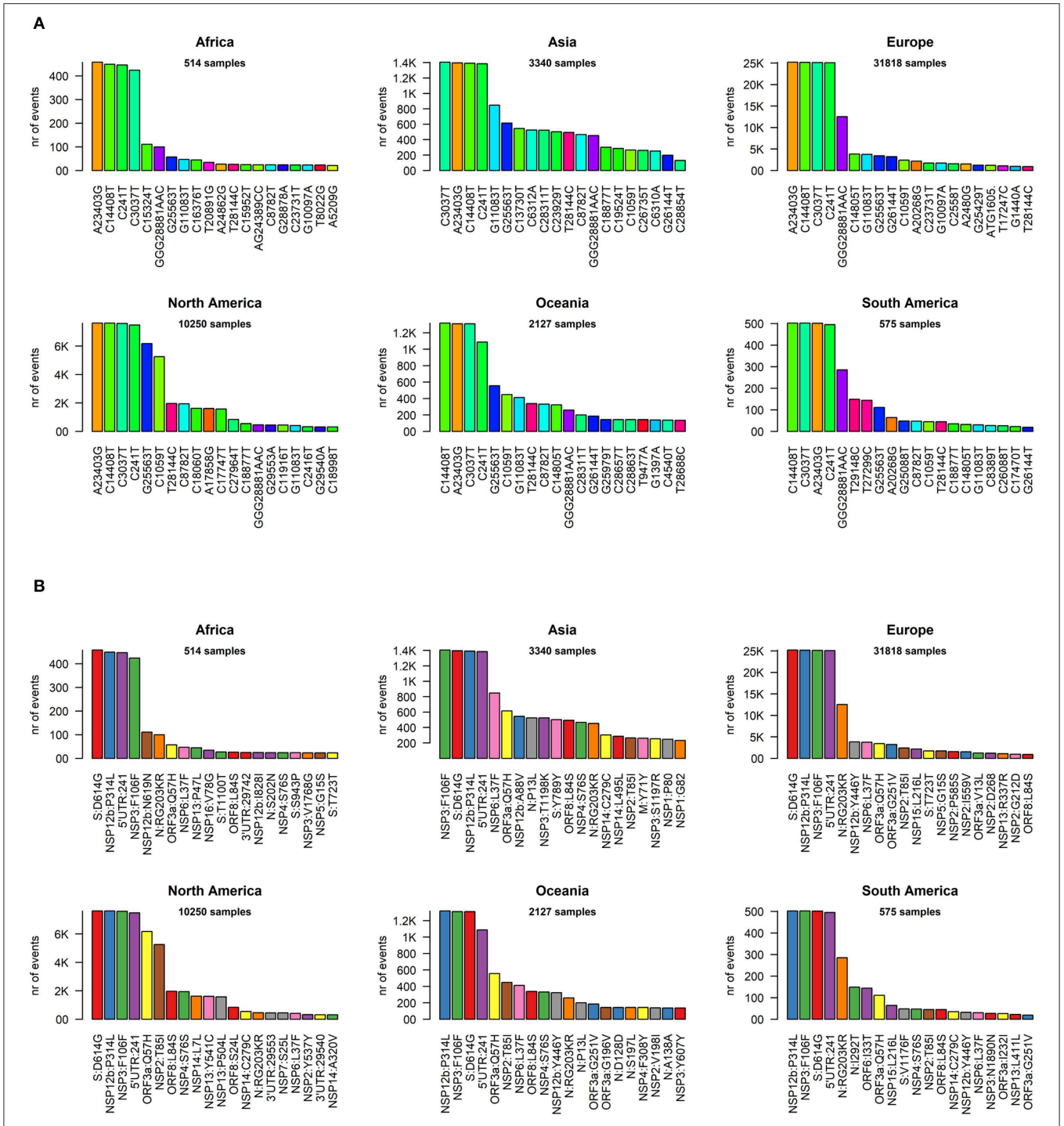


FIGURE 3 | (A) Continent-stratified distribution of SARS-CoV-2 most frequent specific events, annotated as nucleotide coordinates over the reference genome NC_045512.2. Colors are assigned randomly but preserved across panels to facilitate tracking of identical types across continents. **(B)** Continent-stratified distribution of SARS-CoV-2 most frequent specific events, annotated protein changes using the format protein:mutation. Colors are assigned randomly but preserved across panels to facilitate tracking of identical types across continents.

clade GR), characterized by the exclusive presence of the clade-characterizing mutations.

Generally, the G and GR clades are prevalently present in Europe, while the clade S and GH have been mostly observed in the Americas (**Figure 4**). The "L" reference clade is mostly represented by sequences from Asia, where the virus likely originated (Andersen et al., 2020). In **Table 2**, we also report, for reference and completeness, the corresponding nomenclature

TABLE 1 | The 20 most frequent mutation events observed in sequenced SARS-CoV-2 genomes.

Genomic coordinate	Effect on protein/UTR	Nr of samples	Class	Genomic region
A23403G	S:D614G	36,500	aa-changing SNP	Spike protein
C14408T	NSP12b:P314L	36,444	aa-changing SNP	Non-structural protein 12, post-ribosomal frameshift (RNA-dependent RNA polymerase)
C3037T	NSP3:F106F	36,384	silent SNP	Non-structural protein 3 (predicted phosphoesterase)
C241T	5′UTR:241	36,007	5′UTR SNP	5′ UnTranslated Region
GGG28881AAC	N:RG203KR	14,095	aa-changing SNP triplet	Nucleocapsid protein
G25563T	ORF3a:Q57H	10,929	aa-changing SNP	ORF3a protein
C1059T	NSP2:T85I	8,451	aa-changing SNP	Non-structural protein 2
G11083T	NSP6:L37F	5,507	aa-changing SNP	Non-structural protein 6 (transmembrane protein)
C14805T	NSP12b:Y446Y	4,505	silent SNP	Non-structural protein 12, post-ribosomal frameshift (RNA-dependent RNA polymerase)
T28144C	ORF8:L84S	3,804	aa-changing SNP	ORF8 protein
G26144T	ORF3a:G251V	3,792	aa-changing SNP	ORF3a protein
C8782T	NSP4:S76S	3,743	silent SNP	Non-structural protein 4
A20268G	NSP15:L216L	2,479	silent SNP	Non-structural protein 15 (endoRNAse)
C18060T	NSP14:L7L	1,813	silent SNP	Non-structural protein 14 (3′-5′ exonuclease)
C23731T	S:T723T	1,799	silent SNP	Spike protein
G10097A	NSP5:G15S	1798	aa-changing SNP	Non-structural protein 5 (protease)
A17858G	NSP13:Y541C	1,780	aa-changing SNP	Non-structural protein 13
C17747T	NSP13:P504L	1,736	aa-changing SNP	Non-structural protein 13
C2558T	NSP2:P585S	1,701	aa-changing SNP	Non-structural protein 2
A2480G	NSP2:I559V	1,615	aa-changing SNP	Non-structural protein 2

The acronym "aa" stands for "amino acid".

used by the PANGOLIN phylogenetic classification (Rambaut et al., 2020).

Currently, the G clade and its offspring, GH and GR, are the most common clades amongst the sequenced SARS-CoV-2 genomes, globally accounting for 74% of all world sequences (**Figure 5A**). Specifically, the GR clade, carrying the combination of Spike D614G and Nucleocapsid RG203KR mutations, is currently the most common representative of the SARS-CoV-2 population worldwide. The original viral strain, represented by clade L, still accounts for 7% of the sequenced genomes, and the other derived clades S and V have similar frequencies in the global dataset.

At the beginning of the COVID-19 pandemic (December 2019) the most commonly retrieved genome was the reference one (clade L), but the first mutated virus appeared in sequence databases at the beginning of 2020 (clade S) alongside other, less clearly defined, sequences (generic clade O). The clade V (mutated in NSP6 and ORF3a) appeared around mid-January 2020, around the same time as the original clade G (**Figure 5B**). The first detection of subclades GH and GR can be placed more than a month later, at the end of February 2020. Sequencing efforts, mostly located in North America and Europe, have demonstrated an ever-increasing frequency of G, GH, and GR genomes, which have gradually become the most represented sequences in the GISAID database (**Figure 5B**).

Our analysis highlights pivotal differences in clade distribution over time between continents (**Supplementary File 7**, **Figure 5C**). Currently, the vastly prevalent genome in North America is GH (mutations in Spike D614G and ORF3a Q57H), accounting for more than 50% sequences submitted. In Europe and South America, the most frequent clades are GR, while in Oceania there seems to be the most balanced co-existence of all observed clades. Africa shows a prevalence of clade G. It is interesting to note that Asia, initially characterized by reference sequences, is currently observing a rise in G, GH, and GR genomes, which gained ground in the continent at the beginning of March 2020, more than 1 month after the appearance of these clades in Europe (**Figure 5C**).

We provide, as **Supplementary File 8**, also a country-wise analysis of the 32 countries with most SARS-CoV-2 full genome sequences available. As a general observation, countries tend to follow the general trend of their continent, with a few notable exceptions. China, for example, has produced almost no sequences belonging to clades G and derivatives. Moreover, some European countries have a prevalence of GH genomes (Denmark, France), while others show higher numbers of GR (United Kingdom, Portugal). The currently predominant clade in the United States of America is GH, like Israel and Saudi Arabia, while the most common genomes in Russia and Brazil belong to clade GR.

TABLE 2 | Current definition of characterizing mutations of SARS-CoV-2 phylogenetic categorization systems (GISAID clades and PANGOLIN lineages).

GISAID clade	PANGOLIN lineage	Nucleotide features	Corresponding effects on protein sequence
G	B.1	C241T C3037T C14408T A23403G	5′UTR NSP3:F106F NSP12b:P314L S:D614G
GH	B.1.*	C241T C3037T C14408T A23403G G25563T	5′UTR NSP3:F106F NSP12b:P314L S:D614G ORF3a:Q57H
GR	B.1.1	C241T C3037T C14408T A23403G GGG28881AAC	5′UTR NSP3:F106F NSP12b:P314L S:D614G N:RG203KR
S	A	C8782T T28144C	NSP4:S76S ORF8:L84S
V	B.2	G11083T G26144T	NSP6:L37F ORF3a:G251V
L		Reference in all nts defining clades G, GH, GR, S, and V	
O		Others	

Generally speaking, we observe an increase over time in G clade genomes, and its derivatives GH and GR, paired by a gradual disappearance of clades L and V. Clade S, while declining, seems to be still accounting for a significant minority of sequenced genomes, especially in the United States of America and Spain.

As a final part of our analysis, we analyzed the effects of mutations in the 26 SARS-CoV-2 proteins, producing a map of all the most frequent observed aa-changing mutations (**Supplementary File 9**). All proteins are affected by at least one recurring (>75 observations), even if rarer, non-silent mutation. In general, mutations seem to be distributed uniformly across the viral genome, with the obvious exception of highly frequent clade-defining mutations. We analyzed in detail the four structural proteins S (Spike), E (Envelope), M (Membrane), and N (Nucleocapsid) in **Figure 6A**. The Spike protein, apart from the discussed D614G mutation, has no other event present in more than 1% of the viral population; amongst the top 5, a N439K variant located in the Spike/ACE2 interaction domain is observed in 0.7% of the viruses. The Envelope protein appears to be the most conserved, with the most frequent mutations present in the C-terminus and never present in more than 0.2% of the population. More than 1% of sequenced viruses show a T175M mutation in the Membrane protein. The Nucleocapsid protein, apart from the clade GR-defining RG203KR mutation, has several non-silent mutations above the threshold of 1% frequency in the population, specifically P13L, D103Y, S194L, and S197L (**Figure 6A**).

We also analyzed the C241T mutation, located in the SARS-CoV-2 5′UTR. While not inducing a change in protein sequence, we postulated that this event may have effects in the secondary RNA structure, therefore influencing the rate of RNA replication and therefore the speed of the viral infection cycle (Kim et al., 2020). Our prediction, based on the Vienna RNA suite (**Figure 6B**) shows no significant difference in the secondary structure of the wild-type (WT) genome and the C241T variant, since this nucleotide is not participating in any hydrogen bond with other nucleotides.

DISCUSSION

Our analysis, based on 48,635 samples, confirms a low mutation rate of the virus, with an average of 7.23 mutations per sample with respect to the reference SARS-CoV-2 genome sequences. One *caveat* of our estimate is that it is based on assembled genomes, not on raw Illumina, Oxford Nanopore, or Sanger sequencing data. This made it impossible to analyze e.g., the presence of viral subpopulations within the same patient and to evaluate the complex evolutionary events within the SARS-CoV-2 quasispecies (Knyazev et al., 2020). It is therefore likely that the actual mutation rate of SARS-CoV-2 is higher than 7.23, which is calculated from reported sequences of the sole dominant population. This is further sustained by the recent evidence of intense RNA editing in the SARS-CoV-2 genome, fueled by the human host cell APOBEC mechanism (Milewska et al., 2018; Di Giorgio et al., 2020), which can also explain the prevalence of transitions as the prevalently observed mutational events.

While few, the existing detected mutations allow to group the samples into five distinct clades, G, GH, GR, S, and V, characterized by a collection of specific mutations. The clades can be further characterized by most recent mutations and will likely be split even further in the future.

The aa-changing SNPs are the most prevalent mutational events, followed by silent SNPs and extragenic (mostly 5′UTR) SNPs. The silent events may not determine an immediate effect on the protein sequences, but they have repercussions as they may change the codon usage and translation efficiency. In the case of the 5′UTR SNPs, mutations may affect the transcription and replication rates of the virus, or the folding of the genomic ssRNA, processes that have been only recently and only partially elucidated (Kim et al., 2020).

The early studies currently performed on SARS-CoV-2 transcriptome dynamics may also suggest mechanisms for mutation onset, which our study shows being prevalently single-nucleotide transitions. This phenomenon can be associated to defective efficiency of the viral RNA-depedent RNA polymerase or, as recently suggested, by mechanisms of RNA editing triggered by the host cell as a defense mechanism (Di Giorgio et al., 2020). Whatever the origin, SARS-CoV-2 tends to retain its genomic integrity across propagation, with almost no reported large indels across sequenced genomes (the largest reported being

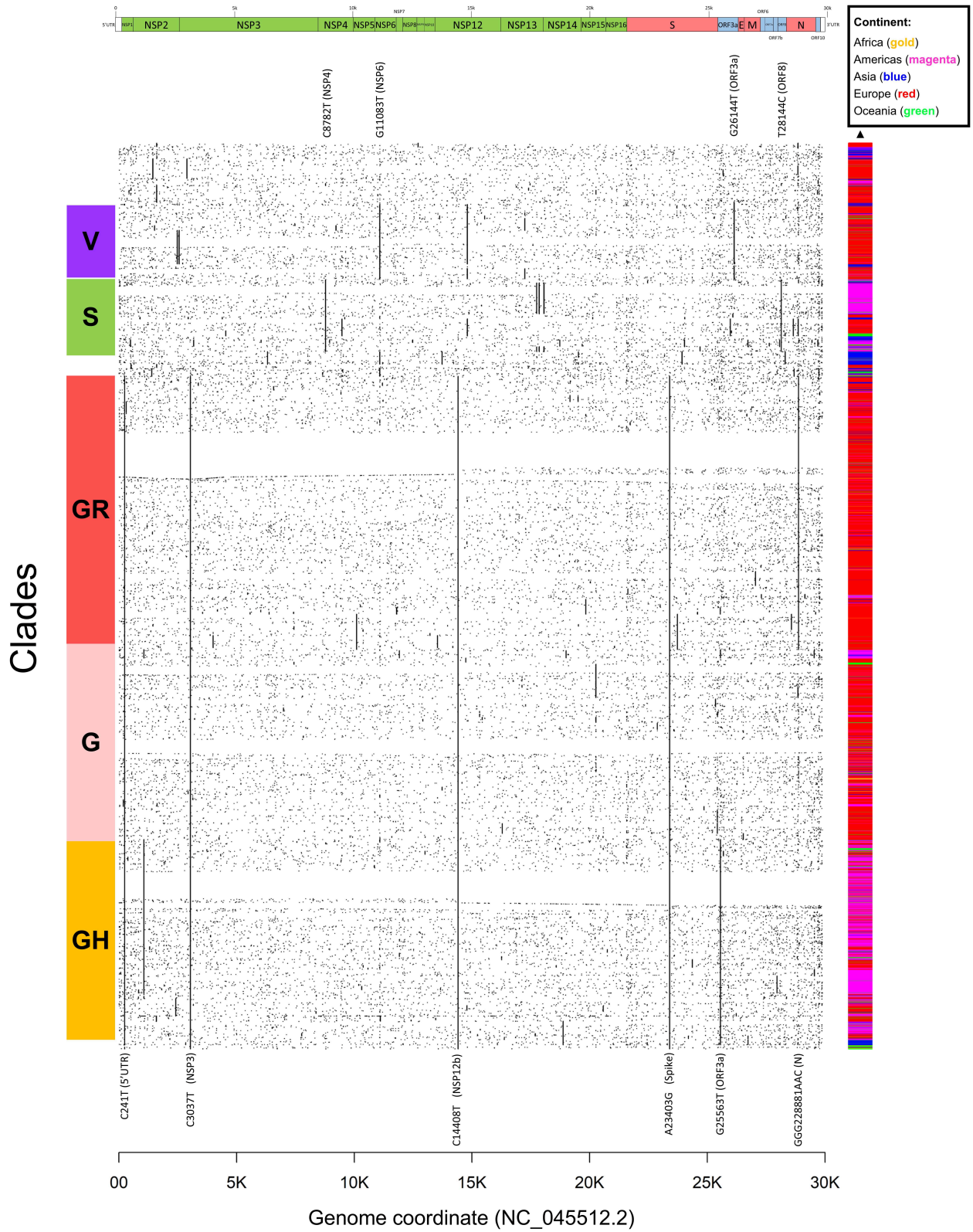


FIGURE 4 | Dot mat showing as X-axis the 29,903 nucleotide positions (sorted from left, 5′ to right, 3′) of SARS-CoV-2, and as Y axis the 48,635 genomes analyzed in this study. The genomic sequences were clustered using simple correlation followed by the "complete" clustering algorithm. Coding sequence regions are shown at the top. To the right of the plot, we assigned a color to each sample according to the continent of origin. On the left, we manually annotated the groups according to the known GISAID clades (G, GH, GR, S, and V) and the mutations that named them. Labels of clade-defining mutations are placed on the corresponding genomic coordinate.

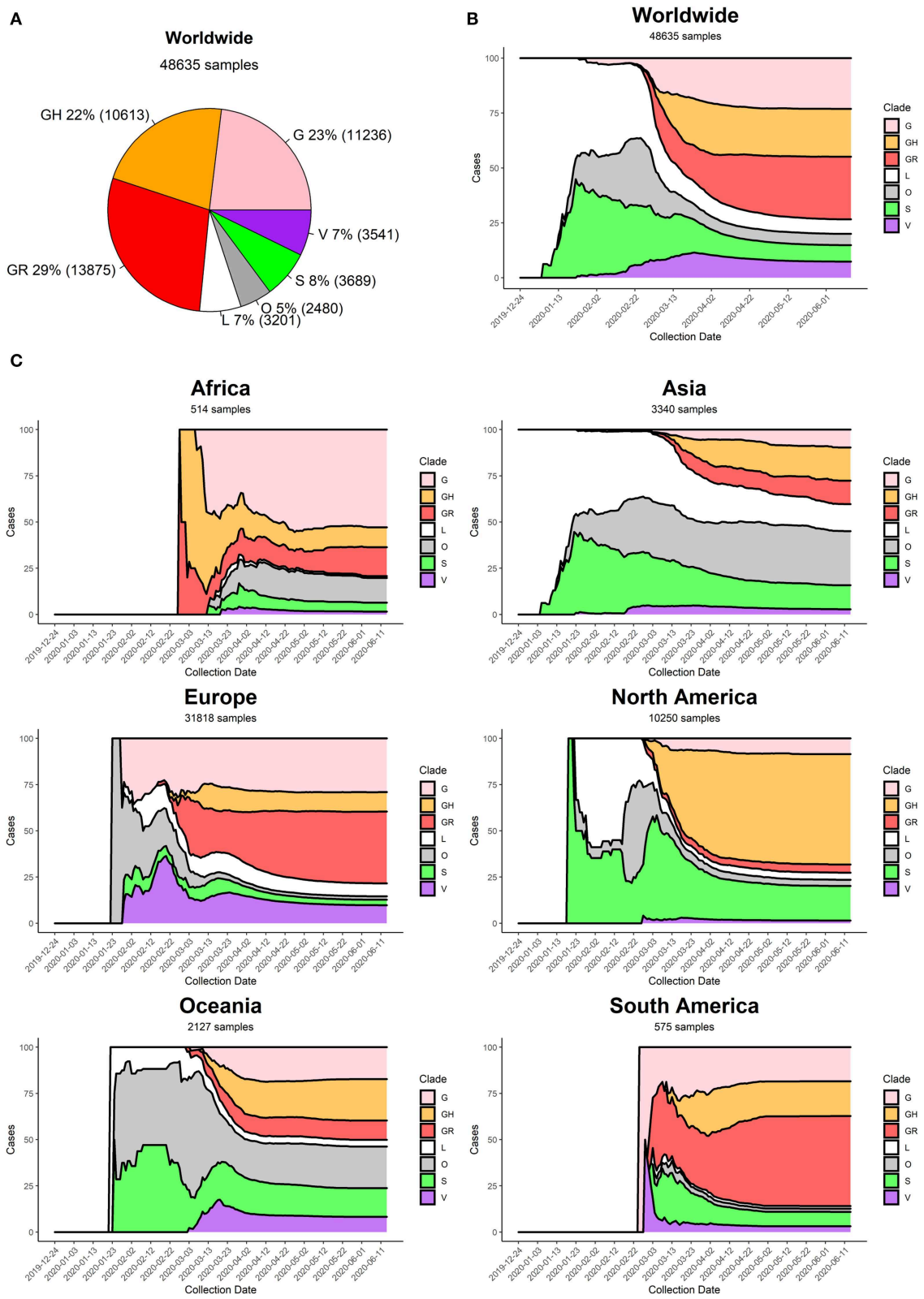


FIGURE 5 | (A) Distribution of SARS-CoV-2 clades in the World at the time of writing (26 June 2020). **(B)** Stacked area chart of relative SARS-CoV-2 clade frequency (y-axis) over time (x-axis) worldwide. **(C)** Stacked area charts of relative SARS-CoV-2 clade frequency over time in six continents.

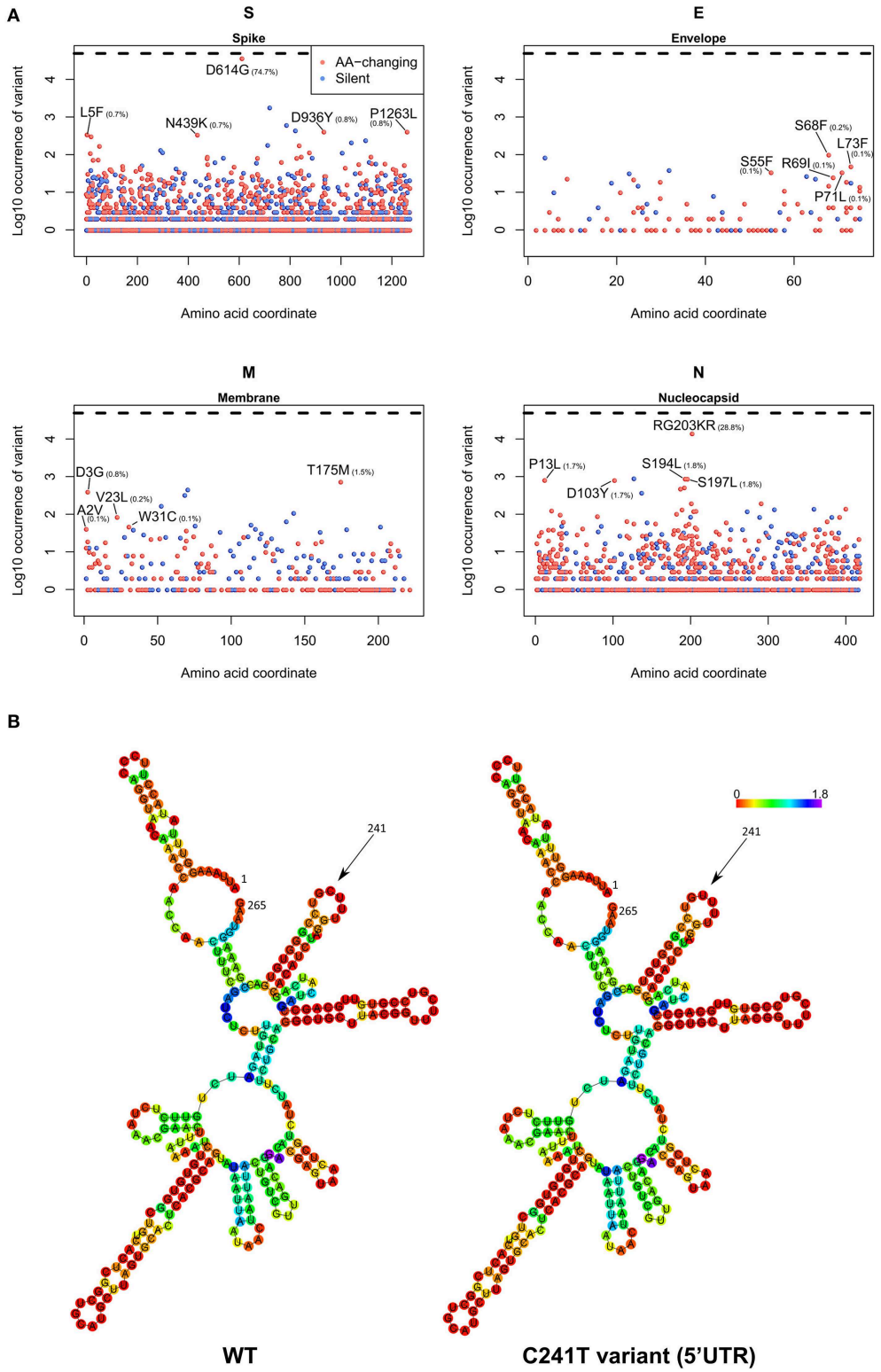


FIGURE 6 | (A) Occurrence of mutations in the four SARS-CoV-2 structural proteins S (Spike), E (Envelope), M (Membrane), and N (Nucleocapsid). On the x-axis, the amino acid coordinate of the mutation. On the y-axis, the Log10 of the number of samples where the mutations have been observed, worldwide. The horizontal dashed line indicates the maximum (Log10 of all the 48,635 samples). In blue, silent mutations, and in red, mutations affecting the protein sequence. The frequency (in percentage) of the top 5 aa-changing mutations is also indicated. **(B)** Dot-bracket notation of minimum free energy prediction of the secondary structure of SARS-CoV-2 5′UTR (nt 1-265), WT (left) and C241T variant (right). Base reliability is expressed as positional entropy and colored accordingly.

a unique 80-nucleotide deletion in ORF7a, in Arizona sample EPI_ISL_424669 – **Supplementary File 5**).

Further studies combining genomic details with epidemiological information and clinical features of COVID-19 patients may be extremely useful to identify strategies and therapies that can help to reduce the burden of this disease. There is currently little evidence on the clinical and molecular differences between the circulating clades of SARS-CoV-2; for example, one study has shown that the D614G mutation in the Spike protein may be associated to higher case fatality rates (Becerra-Flores and Cardozo, 2020). However, as this coronavirus continues to evolve, surely new features will emerge or mutate alongside the genomic sequences, with clinical and pharmacological repercussions.

The emergence of new mutations may force the development of new antiviral therapies, as well as the adaptation of current ones to tackle the new molecular structures of the virus. For example, the development of protein-based and RNA-based vaccines based on the SARS-CoV-2 Spike region (Amanat and Krammer, 2020) will have to take into account the observed diversity of the Spike protein. The prevalent Spike D614G mutation does not seem to affect the interaction domain with ACE2 (Wang et al., 2020), responsible for the viral entry into epithelial cells (Guzzi et al., 2020), but other mutations are currently located in that domain, such as N439K, present in 0.7% of the sequenced SARS-CoV-2 genomes. Our analysis in **Figure 4** shows that new mutations and clades are emerging beyond the current clade categorization and will likely expand if they confer an evolutionary advantage to SARS-CoV-2.

Constant monitoring of mutations will also be pivotal in tracking the movement of the virus between individuals and across geographical areas. For example, our descriptive analysis of clade prevalence over time (**Figure 5**) shows the birth of the original L clade in Asia (China) in December 2019, followed by the appearance of the G clade in Europe in January 2020. G and G-derived clades have then reached North America and Asia in March 2020 and are currently the fastest growing viral subpopulation worldwide. Tracking viral evolution must benefit however from constant monitoring of the SARS-CoV-2 genomic sequences, with *ad-hoc* epidemiological and genomic online resources that go beyond the scope of this publication (Hufsky et al., 2020; Mercatelli et al., 2020). One of such tools is NextStrain (Hadfield et al., 2018), which also allows for scalable phylogenetic analyses and real time tracking of specific mutations.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: https://www.gisaid.org/.

AUTHOR CONTRIBUTIONS

FG designed the study. FG and DM performed research, analyzed data, and wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the Italian Ministry of University and Research, Montalcini Grant 2016, and by CINECA (grant HP10CC5F89).

ACKNOWLEDGMENTS

The authors wish to thank Prof. Michele Morgante and Dr. Davide Scaglione for the fruitful discussions and Dr. Maurizio Grassini and Dr. Giorgio Provenzano for their precious IT support.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.01800/full#supplementary-material

Supplementary File 1 | GISAID acknowledgment table reporting the geographic origin and contributions of genomes analyzed in this study.

Supplementary File 2 | Annotation of samples used in this dataset.

Supplementary File 3 | Annotation of NC_045512.2 SARS-CoV-2 Wuhan genome sequence (GFF3 format).

Supplementary File 4 | Bash/R scripts used to generate and annotate genome variants.

Supplementary File 5 | Full annotation of all mutations identified by this study. Columns are described here. Sample: GISAID sample id; refpos: position in the NC_045512.2 reference genome; refvar: nucleotide composition of the reference at refpos coordinate (a "." indicates an insertion); qvar: variant in the query sample (a "." indicates a deletion); qlength: length of the query genome (reference genome is always 29,903 nucleotides long); region: region annotated in the event position (coding sequence, intergenic or UTR); variant: either a protein change (shown as aminoacid code) or the genomic position (if the event affects a non-coding region); varclass: variant class (as in **Figure 2A**); annotation: full name of the protein coded by the affected region (if coding); varname: full name of protein variant; varclade: full name of nucleotide variant.

Supplementary File 6 | Worldwide analysis of most frequent mutations categorized per class, type, nucleotide, and protein events.

Supplementary File 7 | Distribution of SARS-CoV-2 clades in all continents at the time of writing.

Supplementary File 8 | Stacked area charts of relative SARS-CoV-2 clade frequency (y-axis) over time (x-axis) in the 32 countries with the highest number of full genome sequences.

Supplementary File 9 | Table of aa-changing mutations, categorized by protein and sorted by number of samples where the mutation has been observed.

REFERENCES

Amanat, F., and Krammer, F. (2020). SARS-CoV- 2 vaccines: status report. *Immunity*. 52, 583–589. doi: 10.1016/j.immuni.2020.03.007

Andersen, K. G., Rambaut, A., Lipkin, W. I., Holmes, E. C., and Garry, R. F. (2020). The proximal origin of SARS-CoV-2. *Nat. Med*. 26, 450–452. doi: 10.1038/s41591-020-0820-9

Becerra-Flores, M., and Cardozo, T. (2020). SARS-CoV-2 viral spike G614 mutation exhibits higher case fatality rate. *Int. J. Clin. Pract*. e13525. doi: 10.1111/ijcp.13525. [Epub ahead of print].

Brufsky, A. (2020). Distinct viral clades of SARS-CoV-2: implications for modeling of viral spread. *J. Med. Virol*. doi: 10.1002/jmv.25902. [Epub ahead of print].

Ceraolo, C., and Giorgi, F. M. (2020). Genomic variance of the 2019-nCoV coronavirus. *J. Med. Virol*. 92, 522–528. doi: 10.1002/jmv.25700

Coronaviridae Study Group of the International Committee on Taxonomy of Viruses (2020). The species severe acute respiratory syndrome-related coronavirus: classifying 2019-nCoV and naming it SARS-CoV-2. *Nat. Microbiol.* 5, 536–544. doi: 10.1038/s41564-020-0695-z

Delcher, A. L. (2002). Fast algorithms for large-scale genome alignment and comparison. *Nucleic Acids Res.* 30, 2478–2483. doi: 10.1093/nar/30.11.2478

Di Giorgio, S., Martignano, F., Torcia, M. G., Mattiuz, G., and Conticello, S. G. (2020). Evidence for host-dependent RNA editing in the transcriptome of SARS-CoV-2. *Sci. Adv*. 6:eabb5813. doi: 10.1126/sciadv.abb5813

Dowd, J. B., Andriano, L., Brazel, D. M., Rotondi, V., Block, P., Ding, X., et al. (2020). Demographic science aids in understanding the spread and fatality rates of COVID-19. *Proc. Natl. Acad. Sci. U.S.A*. 117, 9696–9698. doi: 10.1073/pnas.2004911117

Forster, P., Forster, L., Renfrew, C., and Forster, M. (2020). Phylogenetic network analysis of SARS-CoV-2 genomes. *Proc. Natl. Acad. Sci. U.S.A*. 117, 9241–9243. doi: 10.1073/pnas.2004999117

Gruber, A. R., Lorenz, R., Bernhart, S. H., Neuböck, R., and Hofacker, I. L. (2008). The vienna RNA websuite. *Nucleic Acids Res.* 36, W70–74. doi: 10.1093/nar/gkn188

Guzzi, P. H., Mercatelli, D., Ceraolo, C., and Giorgi, F. M. (2020). Master regulator analysis of the SARS-CoV-2/human interactome. *J. Clin. Med.* 9:982. doi: 10.3390/jcm9040982

Hadfield, J., Megill, C., Bell, S. M., Huddleston, J., Potter, B., Callender, C., et al. (2018). Nextstrain: real-time tracking of pathogen evolution. *Bioinformatics* 34, 4121–4123. doi: 10.1093/bioinformatics/bty407

Hu, J., Wang, J., Xu, J., Li, W., Han, Y., Li, Y., et al. (2003). Evolution and variation of the SARS-CoV genome. *Genomics Proteomics Bioinform.* 1, 216–225. doi: 10.1016/S1672-0229(03)01027-1

Hufsky, F., Lamkiewicz, K., Almeida, A., Aouacheria, A., Arighi, C., Bateman, A., et al. (2020). Computational strategies to combat, COVID-19: useful tools to accelerate SARS-CoV-2 and coronavirus research. *Life Sci.* doi: 10.20944/preprints202005.0376.v1

Kim, D., Joo-Yeon, L., Jeong-Sun, Y., Jun Won, K., Narry, K., and Hyeshik, C. (2020). The architecture of SARS-CoV-2 transcriptome. *Cell Press* 181, 914–921.e10. doi: 10.1016/j.cell.2020.04.011

Knyazev, S., Hughes, L., Skums, P., and Zelikovsky, A. (2020). Epidemiological data analysis of viral quasispecies in the next-generation sequencing era. *Brief Bioinform*. bbaa101. doi: 10.1093/bib/bbaa101. [Epub ahead of print].

Lu, R., Zhao, X., Li, J., Niu, P., Yang, B., Wu, H., et al. (2020). Genomic characterisation and epidemiology of 2019 novel coronavirus: implications for virus origins and receptor binding. *Lancet* 395, 565–574. doi: 10.1016/S0140-6736(20)30251-8

Mercatelli, D., Triboli, L., Fornasari, E., Ray, F., and Giorgi, F. M. (2020). Coronapp: a web application to annotate and monitor SARS-Co V-*2* mutations. *bioRxiv [Preprint]*. doi: 10.1101/2020.05.31.124966

Milewska, A., Kindler, E., Vkovski, P., Zeglen, S., Ochman, M., Thiel, V., et al. (2018). APOBEC3-mediated restriction of RNA virus replication. *Sci. Rep*. 8:5960. doi: 10.1038/s41598-018-24448-2

Rambaut, A., Holmes, E. C., Hill, V., O'Toole, Á., McCrone, J., Ruis, C., et al. (2020). A dynamic nomenclature proposal for SARS-CoV-2 to assist genomic epidemiology. *bioRxiv [Preprint].*. doi: 10.1101/2020.04.17.046086

Shen, Z., Xiao, Y., Kang, L., Ma, W., Shi, L., Zhang, L., et al. (2020). Genomic diversity of SARS-CoV-2 in coronavirus disease 2019 patients. *Clin. Infect. Dis.* ciaa203. doi: 10.1093/cid/ciaa203. [Epub ahead of print].

Shu, Y., and McCauley, J. (2017). GISAID: global initiative on sharing all influenza data–from vision to reality. *Eurosurveillance* 22:30494. doi: 10.2807/1560-7917.ES.2017.22.13.30494

Wang, Q., Zhang, Y., Wu, L., Niu, S., Song, C., Zhang, Z., et al. (2020). Structural and functional basis of SARS-CoV-2 entry by using human ACE2. *Cell* 181, 894–904.e9. doi: 10.1016/j.cell.2020.03.045

Wu, C., Liu, Y., Yang, Y., Zhang, P., Zhong, W., Wang, Y., et al. (2020). Analysis of therapeutic targets for SARS-CoV-2 and discovery of potential drugs by computational methods. *Acta Pharm. Sin. B* 10, 766–788. doi: 10.1016/j.apsb.2020.02.008

Zhang, L., Lin, D., Sun, X., Curth, U., Drosten, C., Sauerhering, L., et al. (2020). Crystal structure of SARS-CoV-2 main protease provides a basis for design of improved α-ketoamide inhibitors. *Science.* 368, 409–412. doi: 10.1126/science.abb3405

Zhao, S., and Chen, H. (2020). Modeling the epidemic dynamics and control of COVID-19 outbreak in China. *Quant. Biol.* 8, 11–19. doi: 10.1007/s40484-020-0199-0

Zhou, Y., Hou, Y., Shen, J., Huang, Y., Martin, W., and Cheng, F. (2020). Network-based drug repurposing for novel coronavirus 2019-nCoV/SARS-CoV-2. *Cell Discov*. 6:14. doi: 10.1038/s41421-020-0153-3

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Communication

ORIGINAL RESEARCH
published: 23 July 2020
doi: 10.3389/fcomm.2020.00060

Dissemination Strategies and Usage of Psychological Assistance Hotlines During the COVID-19 Outbreak in China

Ruofan Ma[1,2]*, *Rin Nguyen*[1] and *Jonathan M. Oakman*[2]

[1] Department of Psychology, Michigan State University, East Lansing, MI, United States, [2] Department of Psychology, University of Waterloo, Waterloo, ON, Canada

When the 2019 novel coronavirus disease (COVID-19) was rapidly spreading in China in early 2020, China's National Health Commission quickly responded to the psychological crisis by issuing guidelines for establishing mental health intervention systems, including providing psychological assistance hotlines. However, recent critiques have emphasized China's lack of pre-established mental health interventions which resulted in an inefficient response. This is the first empirical study to systematically examine mental health service use in China during the COVID-19 outbreak. The current study focused on the use of mental health hotlines in a Northern Chinese region. This region originally had a regional level hotline. During the outbreak, 12 out of its 16 sub-regional juridical areas started providing their own hotlines. Data regarding the regional level hotline and the 12 sub-regional level hotlines were obtained, including daily number of calls received, strategies for disseminating hotline services, and callers' expressed concerns. Confirmed COVID-19 cases in China, in the region, and in each of the sub-regional juridical area were also recorded daily during China's peak period of COVID-19. Analyses of these data revealed that the mental health hotlines tended to have low usage overall. Hotlines that merely provided their numbers to community centers and quarantine centers tended to receive few calls. Hotlines that encouraged individuals to advertise the service on personal social media accounts tended to receive more calls. The daily number of confirmed COVID-19 cases in the country was closely related the number of phone calls received at the regional hotline. Sub-regional hotline operators reported that a significant proportion of callers had concerns about contracting COVID-19, negative emotions from prolonged social isolation, and family conflicts while stay-at-home policies were implemented. It was also observed that the sub-regional level hotlines did not start until COVID-19 cases in the country started to decline. Overall, the psychological assistance hotlines provided during COVID-19 satisfied some mental health needs. However, consistent with recent commentaries, the hotline services were not established during the time that demand likely peaked. Future studies are warranted to determine the best strategies to improve the accessibility of mental health hotline services.

Keywords: COVID-19, mental health, dissemination, hotline, service utilization

OPEN ACCESS

Edited by:
Mohan Jyoti Dutta,
Massey University, New Zealand

Reviewed by:
Ravindra Kumar Vemula,
English and Foreign Languages University, India
Victoria Team,
Monash University, Australia

***Correspondence:**
Ruofan Ma
r28ma@uwaterloo.ca

Specialty section:
This article was submitted to Health Communication, a section of the journal Frontiers in Communication

Received: *10 May 2020*
Accepted: *09 July 2020*
Published: *23 July 2020*

Citation:
Ma R, Nguyen R and Oakman JM (2020) Dissemination Strategies and Usage of Psychological Assistance Hotlines During the COVID-19 Outbreak in China. Front. Commun. 5:60. doi: 10.3389/fcomm.2020.00060

INTRODUCTION

In 2019, China was the first country to be affected by the novel coronavirus disease (COVID-19), which would eventually become a worldwide pandemic. The initial COVID-19 outbreak occurred in the city of Wuhan, located in Central China, in December 2019. In January 2020, the number of confirmed cases and deaths due to COVID-19 rapidly increased in China. There were then significant concerns regarding the impact of COVID-19 on the mental health of the Chinese public (e.g., Dong and Bouey, 2020; Liu et al., 2020). Several studies have since reported emotional distress and symptoms of anxiety and depression experienced by people in China (Wang et al., 2020; Xiang et al., 2020; Zhou, 2020). A national study of 52,730 Chinese individuals revealed that 35% experienced psychological distress during the COVID-19 pandemic (Qiu et al., 2020). Soon after COVID-19 started to rapidly spread in China, on January 27th, 2020, The National Health Commission of the People's Republic of China (NHCC) issued guidelines for responding to the psychological crisis and distress created by the disease (National Health Commission of China, 2020c).

However, recent commentaries suggested that the country's organization and management models for psychological interventions still had much to improve (e.g., Dong and Bouey, 2020; Duan and Zhu, 2020; Xiang et al., 2020). Commentaries indicated that the guidelines issued by the NHCC were overly general, providing no specification about how different resources should be delivered to which group of individuals (Dong and Bouey, 2020). Additionally, the practical implementation of mental health services was challenged by unestablished intervention systems and the inadequacy of authoritative mental health organizations (Duan and Zhu, 2020). China lacked a pre-existing and well-established organization and management models for mental health interventions. Therefore, it was relatively difficult for China to efficiently respond to the sudden need for psychological services brought by the COVID-19 crisis. The commentaries provided general directions for establishing and improving mental health response systems in China. For instance, Dong and Bouey (2020) emphasized the importance of proactively establishing community resources, planning for psychological interventions, and implementing preventative strategies before the occurrence of emergency events.

Despite these general recommendations, no empirical studies have systematically examined any type of mental health service in any area of China during the COVID-19 outbreak. Therefore, there are neither specific points of intervention nor steps toward resolution available for Chinese mental health service providers. Chinese mental health service providers are then unable to consider, establish, or improve the system, particularly in the context of crisis response. The service providers are left with the same issue they had faced while responding to the psychological crisis associated with the COVID-19 pandemic, namely, not having specific guidance for a potential next step of implementation.

Psychological assistance hotlines can quickly connect a person in need with a provider. Their implementation formed an important part of the mental health strategy in China during COVID-19 (National Health Commission of China, 2020a,b). The first mental health intervention guidelines issued by the NHCC on January 27th specified that interventions, including psychological assistance hotlines, should be organized at each juridical level (National Health Commission of China, 2020c). On February 2nd, 2020, the NHCC issued a notice for the provinces of China to establish the psychological assistance hotlines (National Health Commission of China, 2020b). The particular guidelines for the establishment followed soon on February 7th, 2020 (National Health Commission of China, 2020a).

Psychological Assistance Hotlines in China

The first psychological assistance hotlines were established in China in the late 1980s (Zhang et al., 1995) (Zhang et al., 1 when it became increasingly common for families to own telephones (e.g.,0.4% of families in Shanghai owned telephones in 1984 compared to 30.3% in 1994; Ji, 1995). The Shanghai Mental Health Hotline (Shanghai is an Eastern-Central Chinese area, within Southern China) was one of the first general psychological assistance hotlines in China. From 1990 to 1992, it received 14,667 calls from all over the country (Ji, 1995). Of these calls, 8,214 had complete records available. An analysis of these records revealed that the majority of calls were concerning intimate or family relationships (Ji, 1995). A significant proportion (12.4%) of the 8,214 calls sought assistance about emotional issues such as depression and anxiety (Ji, 1995). Over the next years, this hotline witnessed an increasing proportion of calls that presented emotional concerns, from 20.3% in 1995 to 31.5% in 1999 (Cheng et al., 2000). An analysis of the Shanghai hotline data over the decade from 1990 to 2000 concluded that hotline counseling provided effective mental health intervention (Zhu et al., 2005).

In addition to general psychological assistance hotlines, the Nanjing Crisis Intervention Center (located in an Eastern-Central Chinese area within Southern China) set up the first crisis intervention hotline in China in 1991 (Xie et al., 1996). This hotline was primarily aimed at suicide prevention. Over the first 4 years of operation, this crisis intervention hotline received over 4,000 calls (Xie et al., 1996).

Few records are available regarding hotline operations in Northern China until early 2000. In 2003, China was faced with the severe acute respiratory syndrome (SARS) epidemic, a viral respiratory illness also caused by a coronavirus. The SARS outbreak was associated with high levels of anxiety and depression among the Chinese public (Liang, 2003), those who were ill with SARS at the time of the study (Cheng and Wong, 2005), and the survivors (Cheng and Wong, 2005; Mak et al., 2009; Moldofsky and Patcai, 2011; Fang et al., 2019). An analysis of a general psychological assistance hotline that mainly received calls from Beijing showed that the hotline provided effective mental health support during the SARS epidemic (Wang et al., 2003). The current study examined psychological assistance hotlines in a Northern Chinese region that operated during the COVID-19 crisis, as directed by relevant NHCC guidelines (National Health Commission of China, 2020a,b,c).

Psychological Assistance Hotlines at the Time of the COVID-19 Outbreak

The geographic region of interest in this study is located in Northern China and is home to ∼16 million people. This Northern Chinese region has 16 sub-regional juridical areas. The specific details of this region (e.g., name of the region, the specific juridical level, dates of its first and last reported COVID-19 case, the precise number of accumulated cases) are omitted to protect the identities of the individuals involved in the original data collection. In this article, "regional level hotline" refers to a hotline established by an organization governed by the regional jurisdiction. A "sub-regional hotline" refers to a hotline established by one of the sub-regional juridical areas within this region. This Northern Chinese region only had one major hotline available at the regional level before the spread of COVID-19[1], which had been available for about 10 years.

This region began to see its first cases of COVID-19 during the last week of January 2020. Following guidelines provided by the NHCC (National Health Commission of China, 2020a,b), 12 out of the 16 sub-regional juridical areas in this region implemented individual hotline services on the same day within the first week of February. The psychological interventions used in this region during the peak of the COVID-19 were typical of similar areas in China (e.g., on February 6th, 2020, Sichuan, a Southwestern Chinese province, started to provide several psychological assistance hotlines to the public; Zhou, 2020). These hotlines were available to address general mental health concerns, not limited to COVID-19 related issues. Operators would provide referrals (e.g., contact information for hospitals) for callers with concerns beyond the hotlines' capacity to support. The hotlines did not offer follow-up calls to the callers. Since these hotlines were not established before the spread of COVID-19 in China, this period provided a unique opportunity to examine factors that may have contributed to the utilization of hotlines at their outset.

Aim and Objectives

The goal of the current study was to characterize usage rates of the hotlines (i.e., number of calls received) in the Northern Chinese region, and to examine factors that may be associated with the usage, such as dissemination strategies and the number of confirmed COVID-19 cases. The first objective of this study was to analyze the amount of usage at these hotlines. Because the sub-regional hotlines were introduced during COVID-19 and needed to be promoted to the public, we predicted that rates of utilization of these hotlines would be low relative to the pre-existing regional hotline. We anticipated that the regional level hotline, which had been established before COVID-19, would receive a greater number of calls.

[1]The only other hotline available in this region before COVID-19 was one offered by the Women's Federation. Data from this hotline were not available for analysis in the current study. However, private communication with the director of this hotline revealed that daily number of phone calls received, before and during COVID-19, were much fewer than the number received by the regional level hotline examined in this study. Not including data from this hotline therefore would unlikely change the results of this study.

The second objective of the study was to describe how the hotlines disseminated their services. Approachability is one factor that may contribute to health service utilization. It relates to the extent to which a health resource is identifiable to people facing health needs (Levesque et al., 2013). One way to increase service approachability is for the services to make themselves known to the people in need. Therefore, this study described how each of the hotlines available in this region advertised its services to the public. We also explored whether and how different methods of service dissemination used by the hotlines might have contributed to their different usage rates.

The third objective of the study was to examine whether the daily number of calls received at the hotlines were related to the daily confirmed number of COVID-19 cases. As a fourth objective, callers' characteristics and concerns raised during the hotline calls were briefly explored. This exploration was to complement the understanding of people who responded to outreach from the hotlines. Understanding hotline users would help target future service outreach and provision.

METHODS

Ethical Considerations

Secondary data obtainment and analyses involved in the current study received ethics clearance through the Institutional Ethics Review Board at the University of Waterloo.

Data Collection

Twelve of the 16 sub-regional juridical areas within the examined region established psychological assistance hotlines during COVID-19. Since the names of the sub-regional areas were not specified in this study to protect the identities of the individuals who provided the information, the areas were randomly numbered and presented as Areas 1–12.

Hotline Service Information and Dissemination Methods

The hours of operation of each hotline was publicly available. All the hotline host organizations were contacted by phone to obtain their organization type (e.g., hospital, counseling service center) and qualification of their hotline operators. The regional level host organization and 10 out of the 12 sub-regional hotline host organizations were able to provide a list of methods used to advertise their own hotline numbers.

Daily Number of Calls Received

The 12 sub-regional hotline host organizations reported the daily number of calls received to the regional hotline host. The regional hotline host was contacted to provide these numbers to the researchers. The number of daily calls obtained was from the date that the sub-regional hotlines were established in the first week of February, till ∼1 week after COVID-19 cases stopped increasing in this entire region. In total, 32 days of data of the daily number of calls received at the 12 sub-regional hotlines were obtained. Since the regional level hotline was previously established, the daily number of calls received was obtained from January 1st, 2020, till April 8th, 2020, for a total of 98 days.

TABLE 1 | Characteristics of hotlines.

Hotline[a]	Total calls[b]	Total calls in proportion to population	Host organization type	Qualification of hotline operators
Regional Hotline	3,206	0.0206%	Psychiatric hospital	Certified psychotherapists
Area 1	191	0.0206%	Private counseling service center	Certified psychotherapists
Area 2	39	0.0045%	Counseling and psychiatry department of hospital	Psychiatrists and certified psychotherapists
Area 3	92	0.0031%	Psychiatric hospital	Psychiatrists
Area 4	1	0.0001%	Government administrative service line	No relevant qualifications
Area 5	18[c]	0.0019%	Psychiatric hospital	Psychiatrists
Area 6	10	0.0029%	Private counseling service center	Certified psychotherapists
Area 7	28	0.0028%	Psychiatric hospital	Certified psychotherapists
Area 8	6	0.0011%	Psychiatric hospital	Psychiatrists
Area 9[d]	10	0.0013%	–	–
Area 10	41	0.0045%	Psychiatric hospital	Certified psychotherapists
Area 11	318	0.0279%	Counseling and psychiatry department of hospital	Psychiatrists
Area 12	5	0.0004%	–	–

[a] *All the hotlines operated 24/7, except for the Area 4 hotline which operated 8:30 am.–5 pm. Monday to Friday.*
[b] *Total number of calls are counted from the day the hotlines stated operating till 1 week after the number of COVID-19 cases stopped increasing in any of the areas. There were 32 days included in total.*
[c] *Most of the phone calls reported by Area 5 were outgoing calls made by hotline operators to individuals at quarantine centers who were identified as having high mental health risk.*
[d] *Data about host organization type and qualification of hotline operators are not available for Area 9 and Area 12.*

Daily Number of Confirmed COVID-19 Cases

Since January 21, 2020, the confirmed COVID-19 cases were reported and updated daily for the public. The research team documented the number of daily confirmed cases for 104 days (from January 21st to May 4th). The daily number of cases at the national level, the region of interest of this study, and the sub-regional areas were noted and used for analyses in the current study.

Content of Phone Calls and Caller Characteristics

In mid-March of 2020, the Area 6 hotline host organization interviewed operators at the regional hotline and at hotlines of Area 1–12 that meet the inclusion criteria described below. The interviews asked (1) about notable demographic characteristics (e.g., age, sex-at-birth) of the people who called, (2) whether the calls received were mostly related to COVID-19, and, (3) what concerns about COVID-19 were raised. Answers to (1) and (2) were noted during the interviews and summarized in writing immediately after the interview. Answers to (3) were transcribed (typed) verbatim during the interviews. The written summaries and interview transcripts were obtained from the Area 6 hotline host to explore the characteristics of people who used the hotlines.

Inclusion and Exclusion Criteria

Only the hotlines that had received a total of more than 10 calls before the end of the first week of March were contacted. Out of the 12 sub-regional hotlines, nine sub-regional hotlines had satisfied the criterion of having received more than 10 calls and were included in the study. Area 5 was excluded because the operator informed the interviewer that the hotline operated mainly by making out-going calls to people at high risk of contracting COVID-19 (see Note 3 of **Table 1**). Area 9 and Area 12 could not be reached and were excluded from this analysis. Two operators at the regional level hotline were interviewed. Therefore, data of caller characteristics and content of calls received were originally collected from nine hotline operators from eight hotlines, including the regional level hotline and seven out of the 12 sub-regional hotlines.

Analyses

Objective 1: Analyzing the Number of Calls Received by the Hotlines During COVID-19

For each of the regional level hotline and the 12 sub-regional hotlines, the calls received at the hotlines were summed across the 32 days during which data of the sub-regional hotlines were obtained. To examine the number of calls in proportion to population size, the sums were then divided into the populations of the areas (i.e., the total number of calls at the regional hotline was divided into the total population of the region; the total number of calls at each sub-regional hotline was divided into the population size of the respective sub-regional area).

Objective 2: Describing Dissemination Strategies of the Hotlines

Descriptions of the dissemination methods were summarized for each hotline. Methods of hotline service dissemination were categorized according to the level of populations reached (e.g., community, individuals).

Objective 3: Examining the Association Between the Number of Calls and the Number of Confirmed COVID-19 Cases

Correlations were calculated between the number of calls received at each hotline and the daily confirmed number of COVID-19 cases in the local area, the entire region, and China

overall. The situation in Hubei and China overall was salient on media and affected policies (e.g., stay-at-home orders, mental health interventions) implemented in regions outside of Hubei. Thus, people's psychological state was likely affected by the severity of COVID-19 both in Hubei locally and in the country overall. The correlational analyses were only done for the regional level hotline and three sub-regional hotlines (i.e., hotlines of Areas 1, 3, and 11). The other sub-regional hotlines received too few calls during the 32 days of hotline operation examined for any correlational results to be interpretable.

Additionally, confirmed cases of COVID-19 were reported since January 21st, 2020, at the national level, before this region saw any COVID-19 cases. The daily number of phone calls received at the regional hotline was obtained until April 8th, 2020. A non-parametric Spearman correlation was calculated for these two variables between these two dates (for a total of 79 days). We also found the period during which calls at the regional hotline peaked, and compared it with the period during which the national daily confirmed COVID-19 cases peaked. To identify the period during which phone calls peaked, we calculated 5-day running averages for the number of phone calls and then subtracted each resulting data point from the next one. The results of the subtractions indicated the *change* in the number of phone calls from day-to-day. A positive change value indicated an increase in the number of calls from the previous day, whereas a negative change value indicated a decrease. We flagged change values whose absolute values were one standard deviation away from the mean. The period between the day with the first flagged positive change and the day with the last negative change was the peak period. When finding the peak period of daily confirmed COVID-19 in the country, two outliers (data from February 12th and 13th) were first removed. The rest of the process of finding the peak was the same as that used for finding the peak period of the regional hotline calls.

Objective 4: Exploring Callers' Characteristics and Concerns Raised During the Calls

The basic characteristics of the callers and their expressed concerns, as described by the hotline operators, were analyzed. The researchers coded the descriptions by categories of concerns (e.g., COVID-19 illness-related anxiety, mental health issues due to prolonged social isolation). Major themes of concerns were summarized.

RESULTS

Representativeness of the Region Studied

The Northern Chinese region studied in the current analyses had a total of ∼150 accumulated COVID-19 cases (0.00087% of the population), with its last confirmed case of COVID-19 reported at the end of February 2020. This total confirmed number was relatively low compared to other areas of the world affected by the COVID-19 pandemic. However, this number is representative of reported COVID-19 cases in areas of China outside of Hubei (the province where Wuhan is located). Specifically, as of the date that this Northern Chinese region saw its last COVID-19 increase, China had accumulated 78,959 COVID-19 cases. Of these cases, 83.48% (65,914 cases;0.11% of the population) were reported within Hubei. The other provincial jurisdictions of China ($n = 33$) had an average number of 395.30 ($SD = 403.4426$) accumulated COVID-19 cases ($M = 0.00092\%$ of population; $SD = 0.00062\%$). Therefore, although the accumulated COVID-19 cases in the region studied appears low compared to other areas of the world, this number is representative of Chinese regions outside of Hubei.

Calls Received at Each Hotline

Characteristics of the hotlines examined (e.g., type of host organization, service hours) are presented in **Table 1**.

Across the 32 days during which data for the sub-regional hotlines were obtained, the number of calls received at the regional level hotline ($n = 3{,}206$) was 0.021% of the population of the region. At the 12 sub-regional level hotlines, the proportion of the number of calls ($M = 63.25$, $SD = 96.59$) to the population size ($M = 1047.91$ thousand, $SD = 651.49$ thousand) in each area ranged from 0.00011 to 0.028%. **Table 1** shows the total number of calls received at each hotline and their proportion to the population. Area 1 (0.021%), Area 11 (0.028%), and the regional level hotline (0.021%) received the highest number of calls in proportion to population size. Although the number of calls ($n = 91$) in Area 3 might seem relatively higher than hotlines in most other areas, its number of calls in proportion to population size (0.0031%) was not as high in rank (e.g., Areas 10 had 41 calls but 0.0045% in proportion to the population size of the area).

Summary

Consistent with prediction, the sub-regional hotlines had low usage. The regional hotline received a relatively large number of calls overall but the usage rate in proportion to population size was still low.

Dissemination Methods of Hotline Service

Dissemination strategies of the hotlines are presented below according to whether the method was through (1) channels owned by the host organization, (2) community channels, (3) targeting specific populations (e.g., people at quarantine centers), (4) reaching individual residents, and (5) methods with non-specific targets. Summaries of these methods are presented in **Table 2**.

Host Organization Channels

The hosts of the regional hotline and the hotlines in Areas 3, 7, and 11 all advertised their hotline numbers through their own official social medial accounts. These hosts also encouraged employees at the organization to re-post the hotline information on their personal accounts. Area 7 host made the re-posting task a requirement for its employees.

All the sub-regional hotline host organizations were also required to submit their hotline numbers to the regional hotline host. On the third day following the start of the sub-regional hotline operations, the regional hotline host posted these numbers along with its own on its official social media account.

TABLE 2 | Hotline Service Dissemination Strategies.

Hotline	Strategies of Service Dissemination			
	Host organization[a]	**Community**	**Targeting specific populations**	**Reaching non-specific populations**
Regional hotline	• Posted on official social media account • Encouraged employees to post hotline information on personal accounts	(Not used)	• Quarantine centers • Corporations • Schools • Hospitals	• Local television channels • Broadcasting
Area 1[b]	(Not used)	(Not used)	• Quarantine centers • Hospitals • Social media accounts of employees' union, women's federation, youth leagues	• Local television channels • Broadcast speakers • Posted on social media accounts of governmental departments
Area 2	(Not used)	• Posted posters • Gave out pamphlets at service booths	• Quarantine centers • Senior care centers • Hospitals • Social media accounts of employees' union, women's federation, youth leagues	(Not used)
Area 3	• Posted on official social media account • Encouraged employees to post hotline information on personal accounts	• Gave out pamphlets at service booths	• Quarantine centers	• Local television channels
Area 5	(Not used)	(Not used)	• Quarantine centers	(Not used)
Area 6	(Not used)	• Gave out pamphlets at service booths	• Quarantine centers	(Not used)
Area 7	• Posted on official social media account • Required employees to post hotline information on personal accounts	(Not used)	• Quarantine centers	(Not used)
Area 8	(Not used)	• Posted on community centers' official social media accounts	• Quarantine centers	(Not used)
Area 10	(Not used)	• Gave out pamphlets at service booths	• Quarantine centers • Entrances of the area	(Not used)
Area 11	• Posted on official social media account • Encouraged employees to post hotline information on personal accounts	• Encouraged community service staff to post on personal social media accounts	• Quarantine centers	(Not used)

[a] *All the sub-regional hotlines reported their phone numbers to the regional level hotline host, and the regional level host posted all the phones numbers on its social media account.*
[b] *Area 1 hotline was the only one which used dissemination strategies that reached individual residents, by delivering text messages through mobile service providers.*

Community Channels

Each sub-regional area had residential communities under its governance. Areas 2, 3, 6, 8, 10, and 11 all advertised their hotline service through community resources. Specifically, posters were posted in the communities of Area 2. Pamphlets were given out at community service booths in Areas 2, 3, 6, and 10. These service booths were operated by volunteers during the COVID-19 to provide body temperature checks. Area 8 had community centers post the hotline number on their official social media accounts. Area 11 encouraged community service staff to post the hotline number on their personal social media accounts.

Targeting Specific Populations

All the hotlines, except for Area 4, provided their numbers to local quarantine centers, where people who had close contact with those who contracted COVID-19 temporarily stayed. The regional hotline additionally provided its number to corporations and schools so they can distribute to individual employees and students. Area 2 provided its numbers to senior care centers. Hotline numbers at the regional level and Areas 1 and 2 were also provided to hospitals under their respective juridical governance. Additionally, Areas 1 and 2 had their hotline numbers posted on social media accounts of the Employees' Union, Women's Federation, and Youth Leagues. Area 10 provided its hotline information to people at entrances of the area to incoming travelers.

Reaching Individuals

Area 1 had all the major mobile service providers forward its hotline number to individual residents through text messages.

TABLE 3 | Correlations between numbers of calls received at selected hotlines and number of days past since their operation, daily confirmed COVID-19 cases in the sub-regional local area, in the region, and in the country.

Hotline	Days past since start of hotline operation		Local reported cases of COVID-19		Regional reported cases of COVID-19		National reported cases of COVID-19	
	$n = 32$	p	$n = 32$	p	$n = 32$[a]	p	$n = 32$	p
Regional hotline	−0.931**	<0.001	–	–	0.802**	<0.001	0.855**	<0.001
Area 1	0.338	0.058	−0.413*	0.019	−0.436*	0.013	0.307	0.087
Area 3	−0.178	0.329	−0.266	0.142	0.234	0.197	0.27	0.135
Area 11	0.772*	0.001	−0.239	0.188	−0.7**	<0.001	−0.789**	<0.001

[a] *The last 8 days had no newly confirmed COVID-19 cases in any area of the region. *$p < 0.05$ and **$p < 0.01$.*

Channels Reaching Non-specific Targets

The number of the regional level hotline and those of Area 1 and 3 were disseminated through local television channels and local newspapers. The regional level hotline was also advertised through broadcasting. Additionally, Area 1 uniquely had broadcast speakers installed throughout the area, and its hotline number was cast to its residents through this channel. Area 1 also had governmental departments post the hotline information on their departmental social media accounts.

Summary

Patterns between methods of service dissemination and hotline usage could be observed. Area 1 used methods that ensure reaching each resident and had relatively high hotline usage. Area 11 received the most phone calls and had the highest number in proportion to population, compared with other sub-regional hotlines. The relatively high usage rate of the Area 11 hotline may be related to its method of service dissemination, as it relied the most heavily on dissemination through individuals' personal social media. Merely relying on dissemination through community centers and/or providing the number to quarantine centers appeared to be inadequate for reaching consumers, as can be seen from Areas 5, 6, and 8. Area 4 did not use any method to advertise its service other than submitting its number to the regional host organization, and hotline at this area had the least number of phone calls. Overall, encouraging individuals to post the hotline service on their social media accounts appeared to be an effective way of service outreach.

Relationship Between Daily Number of Calls and Daily Number of Confirmed COVID-19 Cases

We first observed that Area 1 is the area where COVID-19 cases were concentrated in the region, with 44% of total COVID-19 cases from Area 1 and the rest from the other 16 areas of the region.

Results of the correlational analyses are presented in **Table 3**. Non-parametric Spearman correlations revealed that the calls received at the Area 1 hotline had an increasing trend over time ($r = 0.34$, $df = 30$, $p = 0.058$). The number of calls were negatively correlated with the daily increase of COVID-19 cases in the area ($r = -0.41$, $df = 30$, $p = 0.019$) and in the region overall ($r = -0.44$, $p = 0.013$), but not with the daily increase in the country ($r = 0.31$, $df = 30$, $p = 0.087$).

On the other hand, the number of calls received at the Area 11 hotline significantly increased over time ($r = 0.77$, $df = 30$, $p < 0.001$). The number of calls did not correlate with increases of COVID-19 in this area but had significantly negative correlations with the daily reported of COVID-19 cases in the overall region ($r = -0.70$, $df = 30$, $p < 0.001$) and the country ($r = -0.79$, $df = 30$, $p < 0.001$).

Area 3 saw no trend of the phone calls received at its hotline over time. There was also no relationship between the number of phone calls and the daily confirmed number of COVID-19 in the area, in the region, or the country. As noted previously, although Area 3 received a high number of phone calls that allowed the correlational analysis, the area had a larger population size and the number of phone calls in proportion to population size was low.

At the regional level hotline, the number of phone calls received decreased ($r = -0.93$, $df = 30$, $p < 0.001$) over the 32 days since the sub-regional hotlines started operating. Contrasting the patterns seen in Areas 1 and 3, the number of calls received at the regional hotline positively correlated with daily increase of COVID-19 in the region ($r = 0.80$, $df = 30$, $p < 0.001$) and in the country ($r = 0.86$, $df = 30$, $p < 0.001$). During the 79 days between January 21st and April 8th, the calls at the regional hotline were again significantly positively correlated with the national daily confirmed COVID-19 cases ($r = 0.68$, $df = 30$, $p < 0.001$).

Peak Period of Phone Calls at the Regional Level Hotline

As can be seen in **Figure 1**, the number of phone calls at the regional hotline had a peak period. The beginning of the peak period (i.e., first flagged positive change) occurred on the day after the first day that the region saw its first COVID-19 case. In the following 10 days, the calls showed flagged increases on 9 days, before reaching the absolute peak point and starting to show a decrease in the number of calls (i.e., flagged negative values). We observed that the peak point (i.e., the point whose change from its previous point is positive and whose change to its next point is negative) occurred on the day that the sub-regional hotlines started to operate. There were 16 days between this day of peak and the end of the peak period (i.e., the last

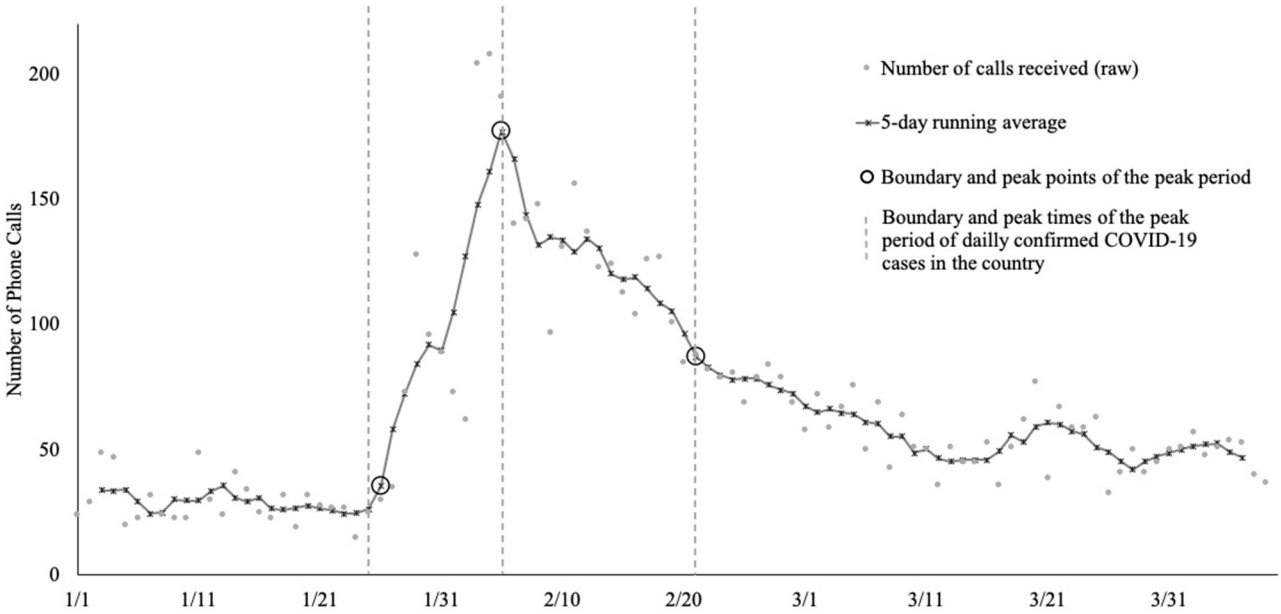


FIGURE 1 | The daily number of phone calls received at the regional hotline from January 1st, 2020, till April 8th, 2020, and 5-day running averages. The number of phone calls showed a monotonic increase since the day COVID-19 cases were reported in this region, peaked on the day the sub-regional hotlines started to operate, and started to show monotonic decrease afterward. The peak period spanned 27 out of the 79 days.

flagged change). Six of the 16 days had a flagged decrease of phone calls and none indicated a flagged increase of calls. The last flagged decrease occurred ∼1 week before the region saw its last COVID-19 increase.

Summary

Taken together, phone calls received at the regional hotline showed a monotonic increase since the day COVID-19 cases were reported in this region, peaked on the day the sub-regional hotlines started to operate, and started to show monotonic decrease afterward. In total, the peak period spanned 27 days. Notably, the number of phone calls received at the regional level hotline appeared to show decreasing trend over time when only examining the calls from the day when the sub-regional hotlines started operation (as described above). However, examining the number of phone calls over a longer period revealed that the number of phone calls changed over time in a non-linear manner.

Peak Period of National COVID-19 Cases

Since the number of COVID-19 cases at the national level correlated with the number of calls received at the regional level hotline, as described above, we also identified the peak period of COVID-19 in China. We observed that the daily increase of COVID-19 cases showed a monotonic increase between January 25th and February 3rd. Changes were not flagged between February 4th and February 6th, and there is a monotonic decrease between February 7th and February 21st, with 10 of the days having flagged negative change values.

We observed that the day on which the regional level hotline calls peaked fell within the days during which the daily confirmed cases of COVID-19 peaked in China, February 3rd to 7th. Interestingly, the day that increase of COVID-19 started to slow down (February 7th) was also the day on which the NHCC issued a specific guideline for establishing mental health hotlines (National Health Commission of China, 2020a). Overall, the peak period of the regional level hotline calls coincided almost perfectly with the peak period of national daily confirmed COVID-19 cases.

Concerns Raised During the Hotline Calls

According to the operators' recollection, callers' ages ranged from seven to over 80 years old, with most individuals between 20 and 50 years old. There were slightly more female callers than males. Major themes of concerns included anxiety about contracting COVID-19, mental health issues associated with social isolation, and conflict with family members due to extended time of staying at home. Quotations illustrating these themes and some other concerns are presented below.

Illness-Related Anxiety

Most calls received during the peak of COVID-19 were related to the crisis. One major theme of concerns revealed was fear of contracting COVID-19:

> Many people were anxious about getting sick, getting the coronavirus. They reported lots of somatized symptoms. It's common to hear reduced appetite and difficulty falling asleep. Also, heart palpitation, increased blood pressure, sweating. People checked their body temperature multiple times a day, staring at the body thermometer all day long. There were also people who repeatedly checked themselves against the coronavirus symptoms.

Social Isolation

Aside from concerns directly related to becoming sick, one major theme of issues was related to long-term isolation. As the hotline operators recalled, many concerns were about not being able to have social interactions and living with the same home routine every day for a prolonged period of time. People started to have feelings of boredom, fatigue, and associated anxiety. For instance, one operator recalled:

> A lot of people were calling because they had to stay at home for this prolonged period. They were in a very different psychological state. Some people used to like playing online games, but then they had this time to play all day long. It became boring and tiring very quickly. Got rid of any gaming addiction right there. Similar to those who watched TV series all day long. There was a lot of anxiety. Anxiety from isolation and not having much to do.

Family Conflict

Another major issue raised was family conflict due to spending extended time with family during isolation at home. As reported by a hotline operator:

> One common issue is family conflict. Before the pandemic, people were busy at work, at school, and family didn't spend so much time together. Now family members were stuck together all day long. Issues that used to be glossed over created explicit conflicts. There were also family conflicts surrounding the coronavirus itself. I remember, to give you an example, one person talked about her mother believing herself being sick and complaining to the rest of the family about not taking her to the hospital. That created a lot of fights.

Other Concerns

The hotline operators also highlighted some issues related to COVID-19 even though they did not form themes as the ones discussed above. For instance, one operator received a call from a 7-year-old child. The child felt helpless because both of the child's parents were working at the medical frontline and the grandparents were quarantined. An elderly caller expressed re-triggered traumatic memories. This caller survived a major earthquake in the 1970s, and the pandemic was triggering anxiety and worry about natural disasters. The hotline operator at Area 1 also highlighted that some callers were survivors of COVID-19 but they felt guilty about having made others sick:

> [Area 1 to this region] is like Wuhan to China. It's where the coronavirus was the worst. Most people got the virus because they went to this one place. Some people who went to this place and then later got tested positive felt very guilty. They felt guilty about having passed the virus to others.

Summary

Overall, hotline operators reported that a significant proportion of hotline callers had concerns directly or indirectly related to COVID-19. However, many calls were about general psychological concerns, not related to COVID-19 per se. The issues related to COVID-19 were grouped into themes including illness-related anxiety, a psychological difficulty about prolonged home isolation, and family discord due to spending more time at home.

DISCUSSION

Despite China's unprecedented emphasis on and speed of response to the psychological crisis when COVID-19 spread in the country, its mental health solutions were criticized as inadequate, primarily due to lack of pre-established organization and management (Dong and Bouey, 2020; Duan and Zhu, 2020). The current study is the first to examine a specific provision of mental health intervention, namely, psychological assistance hotlines, in China during COVID-19. The results revealed the low usage of mental health hotlines during COVID-19. The hotline usage appeared to be related both to ways of service promotion and the number of confirmed COVID-19 cases in the local area and the country. Results from this study suggest specific directions for the improvement of hotline service establishment in China to meet the mental health needs of the public and to prepare for future crises.

Number of Calls Is Associated With Service Dissemination Strategies

The hotline services were used by only a small proportion of the population but some patterns between usage and methods of service dissemination were observed. Hotlines that only relied on disseminating services through community centers (e.g., social media accounts of community centers, community service booths), and/or providing its numbers to local quarantine centers did not receive many calls. The sub-regional hotline that received the highest number of calls relied heavily on advertising its service through personal social media. This hotline encouraged individuals to re-post information about the service on their personal social media accounts. Interestingly, this area was the only one that received a significant increase in phone calls over time. It is possible that this pattern was related to its reliance on social media for service dissemination. It may be that this method allows individuals to directly and quickly share information with others on a platform they are familiar with, rather than relying on the mass public to continuously be aware of and seek out resources provided by community centers. Because individuals continued posting and re-posting the hotline service information within their mutual social circles, the information would be expected to become more widely known as more time passed. This result suggests that where it is consistent with the societal and work culture, using personal social media may be an effective, efficient, and more relatable way of mental health service outreach, reflecting modern forms of communication.

The hotline that reached each individual resident in its service dissemination received a relatively high number of phone calls. However, this area also had the highest number of confirmed COVID-19 cases compared to other sub-regional areas examined. Without another area that experienced a similar impact of COVID-19 but used different service dissemination approaches, it is difficult to dissect the influence of COVID-19 severity from the effectiveness of service outreach.

Notably, although the strategies of service dissemination appeared to be related to the hotline service usage, it is difficult to determine the extent of their causal relationship from the current study. For instance, the area that had the lowest hotline usage barely engaged in service dissemination. The only way it promoted its service to the public was by submitting it to the regional hotline host so the regional host could post it on its social media account, along with phone numbers of the other hotlines. However, this limitation in outreach was not the only downside of the service provided by this hotline. This hotline had the shortest operation hours. It was also the only hotline whose operators did not have training or qualifications to provide psychological interventions. Therefore, although variation in dissemination strategies appeared to be related to variation in hotline usage, this association may be merely a reflection of the general service quality that contributed to service use overall. Future studies are required to determine how different aspects of hotline service provision may influence the number of calls it receives, besides dissemination strategies.

Association of Number of Calls With Confirmed COVID-19 Cases

No consistent patterns were observed across the sub-regional hotlines when comparing the number of calls they received and the number of confirmed COVID-19 cases. However, the number of calls received at the regional level hotline was closely related to the daily confirmed COVID-19 cases reported in the country. The examination of this relationship was possible because the regional level hotline operated prior to the peak of COVID-19 cases in China. The daily number of calls received at the regional level hotline positively correlated with daily confirmed cases reported in the nation. Moreover, the period during which the regional hotline calls peaked coincided almost perfectly with the period during which daily confirmed cases of COVID-19 peaked in China.

Notably, this observed relationship between the number of regional hotline calls and national COVID-19 cases does not preclude the influence of service dissemination on the use of the hotlines. It is possible that dissemination efforts changed over time according to the number of confirmed COVID-19 cases. Specifically, the hotline hosts might have engaged in more outreach when more COVID-19 cases occurred. The effort of service outreach and COVID-19 severity would then affect the number of calls at the same time. Although COVID-19 will become a unique historical event, future studies can experiment with the impact of different service dissemination strategies on the utilization of mental health hotlines in order to better assess causal relationships between dissemination and the utilization of hotline services.

Need for Established Mental Health Systems for Public Crisis Response

Interestingly, the days when the regional level hotline reached its absolute usage peak coincided not only with the days of the absolute peak of daily confirmed COVID-19 in China but also the time when sub-regional hotlines started operating. That is, after the sub-regional hotlines became available, the regional level hotline started to see a decline in the number of phone calls received. It is possible that phone calls that would have been made to the regional hotline were directed to the sub-regional hotlines when these hotlines became available, thus resulting in the decrease in the number of calls to the regional hotline. It would suggest that the sub-regional hotlines did effectively share the service demand that would have been directed to the regional hotline.

The coincidence between the peak of COVID-19 in the country and the beginning of the sub-regional hotline operations revealed potential delays of the hotline service provision. Specifically, the sub-regional hotlines only became available when COVID-19 cases in the country already started to decrease. The services were not established when COVID-19 started to become an increasing concern and when the regional hotline saw increasing usage. This observation is consistent with opinions expressed in the commentaries that criticized the lack of pre-established psychological intervention systems in China, which limited the country's ability to respond effectively when facing crises (Dong and Bouey, 2020; Duan and Zhu, 2020). COVID-19 may be an opportunity for mental health service providers in China to learn from the limitations of the current systems and better prepare for future needs.

Future Development of Hotline Services in China

Over the past decades, psychological assistance hotlines in China have become more available and increasingly accessed (Ji, 1995; Zhu et al., 2005). Even though the public mental health crisis brought by COVID-19 revealed some limitations of the mental health systems in China, the development of mental health systems is a work in progress in every country. In fact, the establishment of psychological assistance hotlines in China during COVID-19 was extremely rapid. The lowest juridical levels set up their hotlines (e.g., the ones examined in the current study) within 2 weeks after the issuance of NHCC's psychological crisis response guidelines (National Health Commission of China, 2020c) and within 1 week after the national notification for hotline establishment (National Health Commission of China, 2020b). Despite such rapid response, service provision still lagged behind the public's service demand (as discussed above). This observation suggests that disaster/crisis response simply cannot rely on the *ad hoc* creation of intervention systems. Mental health systems need to be established in a proactive manner and well-disseminated beforehand, with staff already trained for crisis intervention, in order to effectively serve the need of the pubic when a disaster/crisis occurs.

As mental health service providers continue to improve the quality and accessibility of psychological assistance hotlines, some options may be considered. For instance, the hotlines examined in this study were all general counseling hotlines. The development of hotlines that address specific concerns for

specific populations may help improve the efficiency and quality of service provision. As seen from the themes of COVID-19 related concerns found in this study (i.e., anxiety about disease contraction, the psychological impact of prolonged isolation at home, and family conflicts due to spending more time at home with family), people at different ages experienced a variety of difficulties. The hotline operators, however, might not have been trained to provide services that would suit the diverse needs of the callers. To provide more targeted services, it may be valuable to create hotlines based on the type of issues raised by different populations (e.g., sexual assault; Finn and Hughes, 2008; child abuse; Ngoc, 2005; suicidal ideation; Ohtaki et al., 2016). It should be noted that in countries (e.g., the U.S.) where a variety of hotlines are usually available, it may be difficult for the people in need to identify the hotline that best suits their needs. As hotline providers in China establish their services, it may be beneficial to plan for providing targeted services that are also easily accessible. Future studies could examine how specialty needs are currently addressed and determine whether systematic changes would be required to improve current services.

In addition, the provision of hotline counseling services should be considered in the context of other mental health services available. In some Western countries, hotlines are mainly available for crisis interventions. General counseling services are usually provided by psychotherapists in the community and many consumers have insurance coverage when using these services. The hotlines examined in the current study, similar to many others in China, provide general counseling; in-person consultation from therapists is not always accessible or affordable. Therefore, the establishment of hotline service systems in China may face unique challenges. Chinese mental health providers may need to establish standards suitable for the circumstance of the society, in addition to learning from countries with a longer history of mental health system development.

Challenges in Achieving Service Accessibility

Service dissemination is only one of the many steps involved in translating the needs of mental health care to the actual use of services and attaining desirable health outcomes. Many factors from both the suppliers' and the consumers' side play a role (Ecob and Macintyre, 2000; Andersen, 2008; Andersen et al., 2013; Levesque et al., 2013). For example, Levesque et al. (2013) conceptualized five dimensions that health service providers need to accomplish in order to make the service accessible. These five dimensions included approachability (i.e., the extent to which the service is known and identifiable), acceptability (i.e., the extent to which the service is appropriate for a particular individual in the cultural and social environment), availability and accommodation (i.e., the extent to which the service can be reached in time), affordability (i.e., the extent to which prices and opportunity costs of the service can be covered by one's income) and appropriateness (i.e., the extent to which the service fits the service seeker's needs; Levesque et al., 2013). In reflection to the dimensions presented by Levesque et al. (2013), each sub-regional hotline in the current study made various efforts to increase their approachability by distributing information regarding hotline services.

Corresponding to the five dimensions of accessible service are the five abilities of individuals that would enable their needs to be translated into outcome (Levesque et al., 2013). Namely, these are people's abilities to perceive, seek, reach, pay for, and engage in services. Consumers' ability to perceive the need for care is complementary to approachability of the service provider. For instance, someone who is experiencing anxiety about having COVID-19 may be aware of the hotline resource, but they may not recognize this anxiety as a psychological issue and have no desire to call a mental health hotline. Therefore, it is necessary to increase the mental health literacy of the public so individuals with service needs are able to self-identify and respond to the outreach efforts of the service providers.

Notably, among all the challenges that China needs to overcome for mental health hotlines and other psychological interventions to function adequately, improving service quality may take the longest time and require the most investment. China is currently under a severe shortage of mental healthcare providers (Liang et al., 2018). This shortage was indicated in commentaries about China's mental health provision during COVID-19 (Dong and Bouey, 2020) and is also evident from the varying degree of qualification of mental health hotline operators seen in the current study. The shortage of professionals renders the fast establishment of psychological interventions during crises infeasible (Duan and Zhu, 2020). Consequently, to become effective in mental health service provision, especially when facing unexpected events of crises, China would likely have to engage in long-term commitment and investment in establishing mental health systems.

LIMITATIONS

Several limitations should be noted when interpreting the findings. First, the results of the current study were obtained from the data of one Northern Chinese region. The results may not generalize to other regions, especially those that were geographically further from this region and had different demographics. However, other regions may still be informed by this study because the severity of COVID-19 in this region was on par with most regions in China and the mental health solutions it used were representative of other regions during COVID-19.

Second, observations about mental health hotline usage from this study were made from the limited time period of COVID-19 in China, as the sub-regional hotlines were previously not available. It may inform hotline service usage during a public health crisis. However, before the findings from this study can be used to inform changes to dissemination strategies of mental health hotline services in general, more studies should be done to understand hotline usage outside of the time of a pandemic. For instance, although the operators reported that most individuals who called the

hotlines tended to be between ages 20 and 50, it is unknown whether this age group is also more likely to use hotlines in general.

Third, many factors in addition to service outreach can affect mental health resource utilization. Future studies are warranted to determine to what extent service dissemination can affect usage of mental health hotlines, and what other factors are significant contributors to mental health service use in China, in general, and in response to crises.

Fourth, the qualitative analysis of concerns people expressed during the hotline calls was not systematic or in-depth in this study, due to the limitations of the secondary data that were available. To better understand the issues raised during these calls, future studies should collect systematic records for each call. Finally, the current study did not include information about how various issues were approached by the hotline operators during the calls. As dissemination is only one step in ensuring service use, future studies should examine details of the service provision and understand how it may impact the use of the hotline services. If future studies examine hotline usage post-COVID-19, it may be especially important to accounting for service provision during the crisis, as current service may impact people's trust of the service and therefore future usage.

DATA AVAILABILITY STATEMENT

The data analyzed in this study is subject to the following licenses/restrictions: Hotline usage data used in the study are not shared.

ETHICS STATEMENT

Secondary data obtainment and analyses involved in the current study received ethics clearance through the Institutional Ethics Review Board at the University of Waterloo.

AUTHOR CONTRIBUTIONS

RM conducted the analyses and took the lead in writing the manuscript. JO contributed to conceptualizing study analyses. JO and RN reviewed and provided critical feedback on the manuscript. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

We thank Dr. Amy Drahota for helpful discussions about the idea of the study.

REFERENCES

Andersen, R. M. (2008). National health surveys and the behavioral model of health services use. *Med. Care* 46, 647–653. doi: 10.1097/MLR.0b013e31817a835d

Andersen, R. M., Davidson, P. L., and Baumeister, S. E. (2013). "Improving access to care," in *Changing the U.S. Health Care System: Key Issues in Health Services, Policy, and Management*, ed G. F. Kominski (San Francisco, CA: John Wiley & Sons), 33–69.

Cheng, H., Ji, J. L., and Wen, S. M. (2000). Mental health hotline services in Shanghai between 1995. *Chinese J. Clin. Psychol.* 3. Available online at: http://clinicalpsychojournal.yywkt.cn

Cheng, S. K. W., and Wong, C. W. (2005). Psychological intervention with sufferers from severe acute respiratory syndrome (SARS): lessons learnt from empirical findings. *Clin. Psychol. Psychother.* 12, 80–86. doi: 10.1002/cpp.429

Dong, L., and Bouey, J. (2020). Public mental health crisis during COVID-19 pandemic. *Emerging Infect. Dis.* 26, 1616–1618. doi: 10.3201/eid2607.200407

Duan, L., and Zhu, G. (2020). Psychological interventions for people affected by the COVID-19 epidemic. *Lancet Psychiatry* 7, 300–302. doi: 10.1016/S2215-0366(20)30073-0

Ecob, R., and Macintyre, S. (2000). Small area variations in health related behaviours; do these depend on the behaviour itself, its measurement, or on personal characteristics? *Health Place* 6, 261–274. doi: 10.1016/S1353-8292(00)00008-3

Fang, S., Wang, X. Q., Yang, B. X., Liu, X. J., Morris, D. L., and Yu, S. H. (2019). Survey of Chinese persons managing depressive symptoms: help-seeking behaviours and their influencing factors. *Compr. Psychiatry*, 95, 1–9. doi: 10.1016/j.comppsych.2019.152127

Finn, J., and Hughes, P. (2008). Evaluation of the RAINN national sexual assault online hotline. *J. Technol Human Serv.* 26, 203–222. doi: 10.1080/15228830802094783

Ji, J. (1995). Hotline for mental health in Shanghai, China. *Crisis*, 16, 116–120. doi: 10.1027/0227-5910.16.3.116

Levesque, J. F., Harris, M. F., and Russell, G. (2013). Patient-centred access to health care: conceptualising access at the interface of health systems and populations. *Int. J. Equity Health* 12:18. doi: 10.1186/1475-9276-12-18

Liang, B. (2003). Common psychological stress responses and psycholoigcal interventions during the SARS epidemic in China. *Studies Psychol. Behav.* 3. Available online at: http://psybeh.tjnu.edu.cn/CN/volumn/current.shtml

Liang, D., Mays, V. M., and Hwang, W. C. (2018). Integrated mental health services in China: Challenges and planning for the future. *Health Policy Plan.* 33, 107–122. doi: 10.1093/heapol/czx137

Liu, S., Yang, L., Zhang, C., Xiang, Y. T., Liu, Z., Hu, S., et al. (2020). Online mental health services in China during the COVID-19 outbreak. *Lancet Psychiatry* 7, e17–e18. doi: 10.1016/S2215-0366(20)30077-8

Mak, I. W. C., Chu, C. M., Pan, P. C., Yiu, M. G. C., and Chan, V. L. (2009). Long-term psychiatric morbidities among SARS survivors. *Gen. Hosp. Psychiatry*, 31, 318–326. doi: 10.1016/j.genhosppsych.2009.03.001

Moldofsky, H., and Patcai, J. (2011). Chronic widespread musculoskeletal pain, fatigue, depression, and disordered sleep in chronic post-SARS syndrome: a case-controlled study. *BMC Neurol.* 11:37. doi: 10.1186/1471-2377-11-37

National Health Commission of China (2020a). *Guidelines for Psychological Assistance Hotlines During 2019-nCoV Pneumonia Epidemic*. Hanoi.

National Health Commission of China (2020b). *Notification for Establishing Psychological Assistance Hotlines in Response to 2019-N-Cov Pneumonia Epidemic*.

National Health Commission of China (2020c). *Principles of the Emergency Psychological Crisis Interventions for the New Coronavirus Pneumonia*.

Ngoc, B. (2005). *Hotline Helps Nation's Youth*. Vietnam Investment Review.

Ohtaki, Y., Oi, Y., Doki, S., Kaneko, H., Usami, K., Sasahara, S., et al. (2016). Characteristics of telephone crisis hotline callers with suicidal ideation in Japan. *Suicide Life-Threat. Behav.* 47, 54–66. doi: 10.1111/sltb.12264

Qiu, J., Shen, B., Zhao, M., Wang, Z., Xie, B., and Xu, Y. (2020). A nationwide survey of psychological distress among Chinese people in the COVID-19 epidemic: implications and policy recommendations. *General Psychiatry* 33, 1–3. doi: 10.1136/gpsych-2020-100213

Wang, C., Pan, R., Wan, X., Tan, Y., Xu, L., McIntyre, R. S., et al. (2020). A longitudinal study on the mental health of general population during the COVID-19 epidemic in China. *Brain Behav. Immun.* 87, 40–48. doi: 10.1016/j.bbi.2020.04.028

Wang, Y. N., Gao, W., Bin, and Yang, X. D. (2003). Application assessment of psychological counseling through hotline during the SARS epidemic period. *Chinese J. Behav. Med. Sci.* 5. Available online at: http://zhxwyxynkxzz.yiigle.com

Xiang, Y. T., Yang, Y., Li, W., Zhang, L., Zhang, Q., Cheung, T., et al. (2020). Timely mental health care for the 2019 novel coronavirus outbreak is urgently needed. *Lancet Psychiatry* 7, 228–229. doi: 10.1016/S2215-0366(20)30046-8

Xie, X., Weinstein, L., and Meredith, W. (1996). Hotline in China: one way to help Chinese people. *Psychol. Rep*. 78:90. doi: 10.2466/pr0.1996.78.1.90

Zhang, M., Ji, J., and Chu, Z. (1995). Assessment of Shanghai mental health hotline 上海心理健康热线电话心理咨询应用评估I [In chinese]. 临床精神医学杂志, 5. 195–197.

Zhou, X. (2020). Psychological crisis interventions in Sichuan province during the 2019 novel coronavirus outbreak. *Psychiatry Res.* 286:112895. doi: 10.1016/j.psychres.2020.112895

Zhu, X., Chen, H., and Ji, J. (2005). The analysis of hotline data for mental illness. *Clin. Med. J. China* 2. Available online at: http://www.c-jcm.com/zglcyx/ch/index.aspx

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 23 July 2020
doi: 10.3389/fmicb.2020.01796

Quinolines-Based SARS-CoV-2 3CLpro and RdRp Inhibitors and Spike-RBD-ACE2 Inhibitor for Drug-Repurposing Against COVID-19: An *in silico* Analysis

Rajaiah Alexpandi[1], *Joelma Freire De Mesquita*[2], *Shunmugiah Karutha Pandian*[1] *and Arumugam Veera Ravi*[1]*

[1] Department of Biotechnology, School of Biological Sciences, Science Campus, Alagappa University, Karaikudi, India, [2] Laboratory of Bioinformatics and Computational Biology, Department of Genetics and Molecular Biology, Federal University of Rio de Janeiro State (UNIRIO), Rio de Janeiro, Brazil

OPEN ACCESS

Edited by:
Nejat Duzgunes,
University of the Pacific, United States

Reviewed by:
Fouzia Sadiq,
Shifa Tameer-e-Millat University, Pakistan
Siew Pheng Lim,
Denka Life Innovation Research (DLIR), Singapore

***Correspondence:**
Arumugam Veera Ravi
aveeraravi@rediffmail.com

Specialty section:
This article was submitted to Virology, a section of the journal Frontiers in Microbiology

Received: 12 May 2020
Accepted: 09 July 2020
Published: 23 July 2020

Citation:
Alexpandi R, De Mesquita JF, Pandian SK and Ravi AV (2020) Quinolines-Based SARS-CoV-2 3CLpro and RdRp Inhibitors and Spike-RBD-ACE2 Inhibitor for Drug-Repurposing Against COVID-19: An in silico Analysis. Front. Microbiol. 11:1796. doi: 10.3389/fmicb.2020.01796

The novel coronavirus SARS-CoV-2 disease "COVID-19" emerged in China and rapidly spread to other countries; due to its rapid worldwide spread, the WHO has declared this as a global emergency. As there is no specific treatment prescribed to treat COVID-19, the seeking of suitable therapeutics among existing drugs seems valuable. The structure availability of coronavirus macromolecules has encouraged the finding of conceivable anti-SARS-CoV-2 therapeutics through *in silico* analysis. The results reveal that quinoline,1,2,3,4-tetrahydro-1-[(2-phenylcyclopropyl)sulfonyl]-trans-(8Cl) and saquinavir strongly interact with the active site (Cys-His catalytic dyad), thereby are predicted to hinder the activity of SARS-CoV-2 3CLpro. Out of 113 quinoline-drugs, elvitegravir and oxolinic acid are able to interact with the NTP entry-channel and thus interfere with the RNA-directed 5′-3′ polymerase activity of SARS-CoV-2 RdRp. The bioactivity-prediction results also validate the outcome of the docking study. Moreover, as SARS-CoV-2 Spike-glycoprotein uses human ACE2-receptor for viral entry, targeting the Spike-RBD-ACE2 has been viewed as a promising strategy to control the infection. The result shows rilapladib is the only quinoline that can interrupt the Spike-RBD-ACE2 complex. In conclusion, owing to their ability to target functional macromolecules of SARS-CoV-2, along with positive ADMET properties, quinoline,1,2,3,4-tetrahydro-1-[(2-phenylcyclopropyl)sulfonyl]-trans-(8Cl), saquinavir, elvitegravir, oxolinic acid, and rilapladib are suggested for the treatment of COVID-19.

Keywords: drug repurposing, SARS-CoV-2, main-protease (3CLpro), RNA-dependent RNA-polymerase, spike-ACE2 complex, quinoline based-drugs

INTRODUCTION

The current outbreak of coronavirus disease 2019 (COVID-19), caused by the severe acute syndrome coronavirus-2 (SARS-CoV-2), has been considered as a major anxiety of the twenty first century (Dey et al., 2020). As of May 07, 2020, WHO states that over 3,672,238 cases have been authoritatively affirmed, including 254,045 deaths around the globe. The pathognomonic

symptoms of COVID-19 are fever, dry cough, shortness of breath, and dyspnea (Wu et al., 2020). In severe conditions, it causes hypercytokinemia, lymphopenia, disseminated intravascular coagulation, severe acute respiratory syndrome, kidney failure, and eventually death (Thomas-Rüddel et al., 2020). In general, SARS-CoV-2 is a positive sense, long (30,000 bp), single-strand RNA coronavirus that belongs to the family *Coronaviridae* and genus *Betacoronavirus*, which is highly similar to SARS-CoV (Zou et al., 2020). No specific medication for COVID-19 is accessible at the present time. Thus, researchers are seriously searching for suitable vaccines and therapeutic-drugs against COVID-19 (Yao et al., 2020). The fact is that discovery, as well as marketing, of new drugs frequently takes months to years (Stebbing et al., 2020), and hence looking for appropriate therapeutics among existing-drugs seems to be a promising strategy to control the current pandemic of COVID-19 in this critical time.

In the SARS-CoV-2 macromolecules, the large-polyproteins-encoded cysteine protease, called 3-chymotrypsin like protease [3CLpro or main protease (Mpro)], are essential for the viral life-cycle of novel coronavirus (Zhang H. et al., 2020). This enzyme plays a crucial role in the processing of viral polyproteins, which are indispensable for viral maturation and their infectivity (Khan et al., 2020). Subsequently, RNA-dependent RNA polymerase (RdRp) is a key enzyme essential for the viral replication of SARS-CoV-2 (Gao et al., 2020). Due to their crucial roles, these viral proteins are considered as imperative targets for developing antiviral compounds against COVID-19 (Wu et al., 2020). Recently, Choy et al. (2020) reported that the combination of HIV-protease inhibitors such as lopinavir/ritonavir effectively kills SARS-CoV-2 at the cellular level. Similarly, Wang M. et al. (2020) reported that the nucleotide analog RdRp-inhibitor, remdesivir, successfully inhibited SARS-CoV-2 *in vitro*. Hall and Ji (2020) reported zanamivir, indinavir, saquinavir, and remdesivir as SARS-CoV-2 3CLpro inhibitors using *in silico* analysis. Further, Elfiky (2020) also suggested ribavirin, remdesivir, sofosbuvir, galidesivir, and tenofovir as potent drugs against SARS-CoV-2 through docking analysis.

On the other hand, human angiotensin-converting enzyme 2 (ACE-2), a type-I integral membrane protein, has been considered to be the specific and functional receptor for the spike glycoprotein of SARS-CoV-2 (Patel et al., 2014). It is also well-known to play the main role in the rennin-angiotensin system (RAS), which is associated with the regulation of heart function and blood pressure hemeostasis (Oudit et al., 2003). The coronavirus entry into host cells is mediated by the spike glycoprotein, which is a surface transmembrane protein in SARS-CoV-2 (Zhao et al., 2020). The analysis of the receptor-binding motif (RDM) in the Spike glycoprotein revealed that most of the aminoacid residues essential for receptor-binding with ACE-2 were conserved between SARS-CoV-2 and SARS-CoV, demonstrating that these viruses use the same host receptor for cell entry (Yan et al., 2020). Hoffmann et al. (2020) proved that anti-human ACE-2 antibody (R&D Systems, Catalog #AF933) can inhibit the Spike protein-associated entry into cultured cells *in vitro*. Accordingly, human ACE2 is considered as a host target for the treatment of COVID-19 to avoid SARS-CoV-2 from entering host cells (Zhang L. et al., 2020).

The existing quinoline-based antimalarial drugs, hydroxychloroquine and chloroquine, have shown their potential in the treatment of COVID-19 (Kaur et al., 2010), which inspired us to identify the quinoline-based potent inhibitors against the therapeutic targets of SARS-CoV-2 using an *in silico* approach. Due to the drug-like properties and therapeutic potential, quinoline-derived compounds have sustained attention for developing novel drugs in future medicine (O'donnell et al., 2010). Quinolines are nitrogen-containing heterocyclic aromatic compounds, known to be versatile compounds because of their extensive uses in medicine, organic chemistry, and industrial chemistry (Prajapati et al., 2014). They are frequently found in several medicinal plants and are known to have antimalarial, anticancer, antibacterial, anti-fungal, anticonvulsant, anti-inflammatory, anthelminitc, cardiotonic, and analgesic activity (Hussaini, 2016). Some of the compounds with quinoline core are the preferred choice for the treatment of diverse ailments, especially cancer and malaria (Touret and de Lamballerie, 2020).

MATERIALS AND METHODS

Ligand Preparation

Numerous medicinal plants and their phytocompounds have demonstrated their antiviral properties against a large group of viruses. Consequently, the phytocompounds of *Diplocyclos palmatus* leaf extract were subjected to docking analysis in the current study. In previous studies, the tropical medicinal plant of *D. palmatus* has been reported for its anti-biofilm, anti-infection, and anti-photoaging activity using *Caenorhabditis elegans* model (Alexpandi et al., 2019). The list of quinoline-drugs (total 113) was retrieved from DrugBank database (https://www.drugbank.ca/categories/DBCAT000788). The canonical SMILES of the compounds was retrieved from the PubChem database. The canonical SMILES of quinoline,1,2,3,4-tetrahydro-1-[(2-phenylcyclopropyl)sulfonyl]-trans-(8CI) was retrieved from the Guidechem database (https://www.guidechem.com/reference/dic-395649.html). Then, the PDB-format 3D-structure of compounds was downloaded from the Openbabel online server http://www.cheminfo.org/Chemistry/Cheminformatics/FormatConverter/index.html.

Protein Preparation

The 3D crystal protein-structures of SARS-CoV-2 3CLpro (PDB ID: 6LU7) (Hall and Ji, 2020), SARS-CoV-2 spike protein-ACE-2 receptor-binding domain (RBD) (PDB ID: 6M17) (Wu et al., 2020), and human ACE2 (PDB ID: 1R4L) (Joshi et al., 2020) were obtained from the RCSB PDB database (http://www.rcsb.org/pdb). The 3D crystal structures of SARS-CoV-2 RdRp generated through homology modeling using ICM 3.7.3 modeling software was gifted by Prof. Hua Li, Hubei Key Laboratory of Natural Medicinal Chemistry and Resource Evaluation, School of Pharmacy, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China (Wu et al., 2020). The energy minimization of targeted protein structures was

performed using the YASARA server. The protein preparation was done with AutoDock Tools Version 1.5.6.

Molecular Docking

The virtual screening of best scoring compounds was performed using the iGEMDOCK with blind-mode docking. The iGEMDOCK tool is a graphical-automatic drug design system mainly used for structure-based virtual screening of drug molecules (Hsu et al., 2011). After the selection of best binding compounds, the interaction on the active domains of therapeutic targets (3CLpro, RdRp, and Spike-ACE2 complex) of selected compounds were analyzed using the AutoDock Vina tool. It is an open-source docking software, which extensively improves the average accuracy of the binding mode predictions of compounds better than other docking tools (Trott and Olson, 2010). It implements a competent optimization algorithm for estimating the affinity of protein-ligand interactions and predicting the plausible binding modes of compounds (Goodsell et al., 1996). Then, the ligand-protein interactions were visualized by Maestro 10 (Schrödinger) (Balasubramaniam et al., 2019). In the present study, to compare the selected quinolines with already reported anti-SARS-CoV-2 drugs, lopinavir (Hall and Ji, 2020) and remdesivir triphosphate (Gordon et al., 2020) were selected as positive inhibitors of SARS-CoV-2 for *in silico* analysis in the present study. Further, the selected quinolines and remdesivir triphosphate were compared with the parental nucleotides (NTPs) of SARS-CoV-2 RdRp for understanding the inhibition mode of viral replication.

In silico Drug-Likeliness and Bioactivity Prediction

The drug likeliness and bioactivity of quinolines were analyzed using the Molinspiration server (http://www.molinspiration.com). Molinspiration tool is a cheminformatics software that provides molecular properties as well as bioactivity prediction of compounds (Mabkhot et al., 2016). In the Molinspiration-based drug-likeness analysis, there are two important factors, including the lipophilicity level (log *P*) and polar surface area (PSA) directly associated with the pharmacokinetic properties (PK) of the compounds (Beetge et al., 2000). In the Molinspiration-based bioactivity analysis, the calculation of the bioactivity score of compounds toward GPCR ligands, ion channel modulators, kinase inhibitors, nuclear receptor ligands, protease inhibitors, and other enzyme targets were analyzed by sophisticated Bayesian statistics (Mabkhot et al., 2016). This was done as the protein families, such as G protein-coupled receptors (GPCR), ion channels, kinases, nuclear hormone receptors, proteases, and other enzymes (RdRp), are the major drug targets of most of the drugs (Hauser et al., 2017).

In silico ADMET Analysis

The PK properties, such as Absorption, Distribution, Metabolism, Excretion, and Toxicity (ADMET), of quinolines were predicted using the admerSAR v2.0 server (http://lmmd.ecust.edu.cn/admetsar2/). The admerSAR server is an open-source computational tool for prediction of ADMET properties of compounds, which makes it a practical platform for drug discovery and other pharmacological research (Guan et al., 2019). In the ADMET analysis, the absorption (A) of good drugs depends on factors such as membrane permeability [designated by colon cancer cell line (Caco-2)], human intestinal absorption (HIA), and the status of either P-glycoprotein substrate or inhibitor. The distribution (D) of drugs mainly depends on the ability to cross the blood-brain barrier (BBB). The metabolism (M) of drugs is calculated by the CYP, MATE1, and OATP1B1-OATP1B3 models. Excretion (E) of the drugs is estimated based on the renal OCT substrate. Then, the toxicity (T) of the drugs is predicted on the Human Ether-a-go-go- related gene inhibition, carcinogenic status, mutagenic status, and acute oral toxicity (Shen et al., 2012).

RESULTS

Screening of Potent SARS-CoV-2 3CLpro Inhibitors

In **Table S1**, the iGEMDOCK-based virtual screening result reveals that among the 17 phytocompounds, the novel quinoline (NQ) identified from *D. palmatus,* quinoline,1,2,3,4-tetrahydro-1-[(2-phenylcyclopropyl)sulfonyl]-trans-(8CI), has a lower binding energy (-6.8 Kcal/mol) with SARS-CoV-2 3CLpro, compared to other phytocompounds. As shown in **Figures 1D,I**, the NQ builds five hydrophobic interactions (Cys145, Met49, Met165, Phe140, and Leu141), 8 polar interactions (His41, His164, Gln192, Gln189, His63, His172, Ser144, and Asn142), two negative-charged interactions (Asp187 and Glu166), and one unspecified residue interaction (Arg188). As shown in **Figure 1G**, the NQ forms a hydrogen bonding interaction with Glu166 (2.044 Å distance). Importantly, the NQ has effectively interacted with the active site of catalytic-dyad (Cys145 and His41) of the SARS-CoV-2 3CLpro (**Figures 1B,F**), and hence we assumed that the NQ is able to hinder the protease activity of SARS-CoV-2 like lopinavir (−6.6 Kcal/mol) (**Figure S1**), which is a protease-inhibitor based anti-SARS-CoV-2 drug reported for COVID-19 (Choy et al., 2020).

Based on the virtual screening results of SARS-CoV-2 3CLpro, rilapladib, saquinavir, oxolinic acid, elvitegravir, batefenterol, sitafloxacin, CP-609754, GSK-256066, alatrofloxacin, and quarfloxin were predicted to be the best compounds from the commercial quinoline-based drugs (**Figure 2** and **Table S2**). Out of these compounds, only saquinavir (−8.5 Kcal/mol) was found to interact with the active catalytic-domain (Cys145 and His41) of the SARS-CoV-2 2CLpro (**Figures 1H,C**). As shown in **Figures 1E,J**, saquinavir forms seven hydrophobic interactions (Cys145, Met165, Met49, Leu27, Leu167, Leu141, and Phe140), 12 polar interactions (His41, Asn142, His163, Ser144, Ser46, Thr25, Thr26, Thr24, Gln192, Gln189, Thr190, and His164), one negative-charged interaction (Glu166), and one glycine interaction (Gly143) with SARS-CoV-2 3CLpro. Therefore, for the development of strong interactions at the CysHis catalytic dyad, saquinavir was predicted to obstruct the 3CLpro activity.

Screening of Potent SARS-CoV-2 RdRp Inhibitors

As shown in **Figure 3** and **Table S3**, amongst the tested-quinolines, elvitegravir, oxolinic acid, saquinavir, garenoxacin,

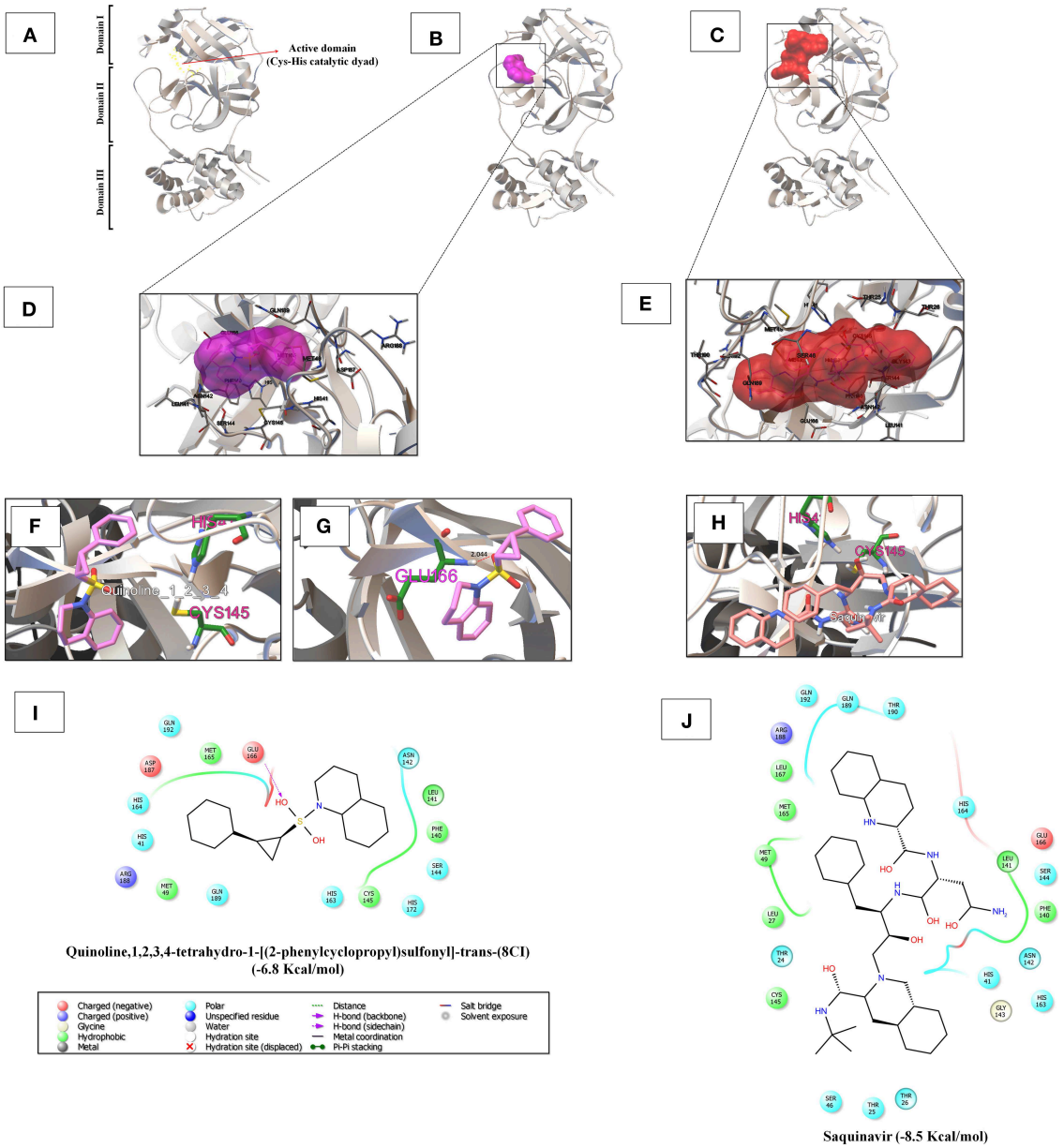


FIGURE 1 | (A) The 3CLpro monomer has three domains, namely domain I (residues 8–101), domain II (residues 102–184), and domain III (residues 201–303). The red arrow mark indicates the region of the active site of 3CLpro, which is located in the gap between domains I and II, and has a CysHis catalytic dyad (Cys145 and His41). **(B,C)** Depicts the binding region of the quinoline,1,2,3,4-tetrahydro-1-[(2-phenylcyclopropyl)sulfonyl]-trans-(8Cl) (−6.8 Kcal/mol) and saquinavir (−8.5 Kcal/mol) with SARS-CoV-2 3CLpro, respectively. **(D,E)** Shows the super-position view of the binding sites and their interacting amino acids of quinoline,1,2,3,4-tetra hydro-1-[(2-phenylcyclopropyl)sulfonyl]-trans-(8Cl) and saquinavir with SARS-CoV-2 3CLpro, respectively. **(F,H)** Indicates the interaction with the active catalytic dyad of SARS-CoV-2 3CLpro (Cys145 and His41). **(G)** Hydrogen bond formation of quinoline,1,2,3,4-tetrahydro-1-[(2-phenylcyclopropyl)sulfonyl]-trans-(8Cl) with Glu166 residues of 3CLpro at 2.044 Å distance. **(I,J)** Reveal the interacted aminoacid residues of SARS-CoV-2 3CLpro with quinoline,1,2,3,4-tetrahydro-1-[(2-phenylcyclopropyl)sulfonyl]-trans-(8Cl) and saquinavir.

rilapladib, pelitinib, difloxacin, batefenterol, danofloxacin, and LGD-2226 were shown to be the best-docked compounds against SARS-CoV-2 RdRp. **Figures 4A,C** reveals that elvitegravir and oxolinic acid have the same binding energy (−7.1 Kcal/mol) and same binding site at SARS-CoV-2 RdRp, which is almost similar to natural nucleotides such as ATP (−7.6 Kcal/mol), UTP (−7.1 Kcal/mol), GTP (−7.7 Kcal/mol), and CTP (−7.1 Kcal/mol) (**Figure S2**). As shown in **Figures 4B,I**, elvitegravir forms five hydrophobic interactions (Tyr455, Ala554, Tyr619, Pro620, and Cys622), seven polar interactions (Thr556, Thr680, Ser681, Ser682, Thr687, Asn691, and Ser759), four negative-charged interactions (Asp452, Asp618, Asp623, and Asp760),

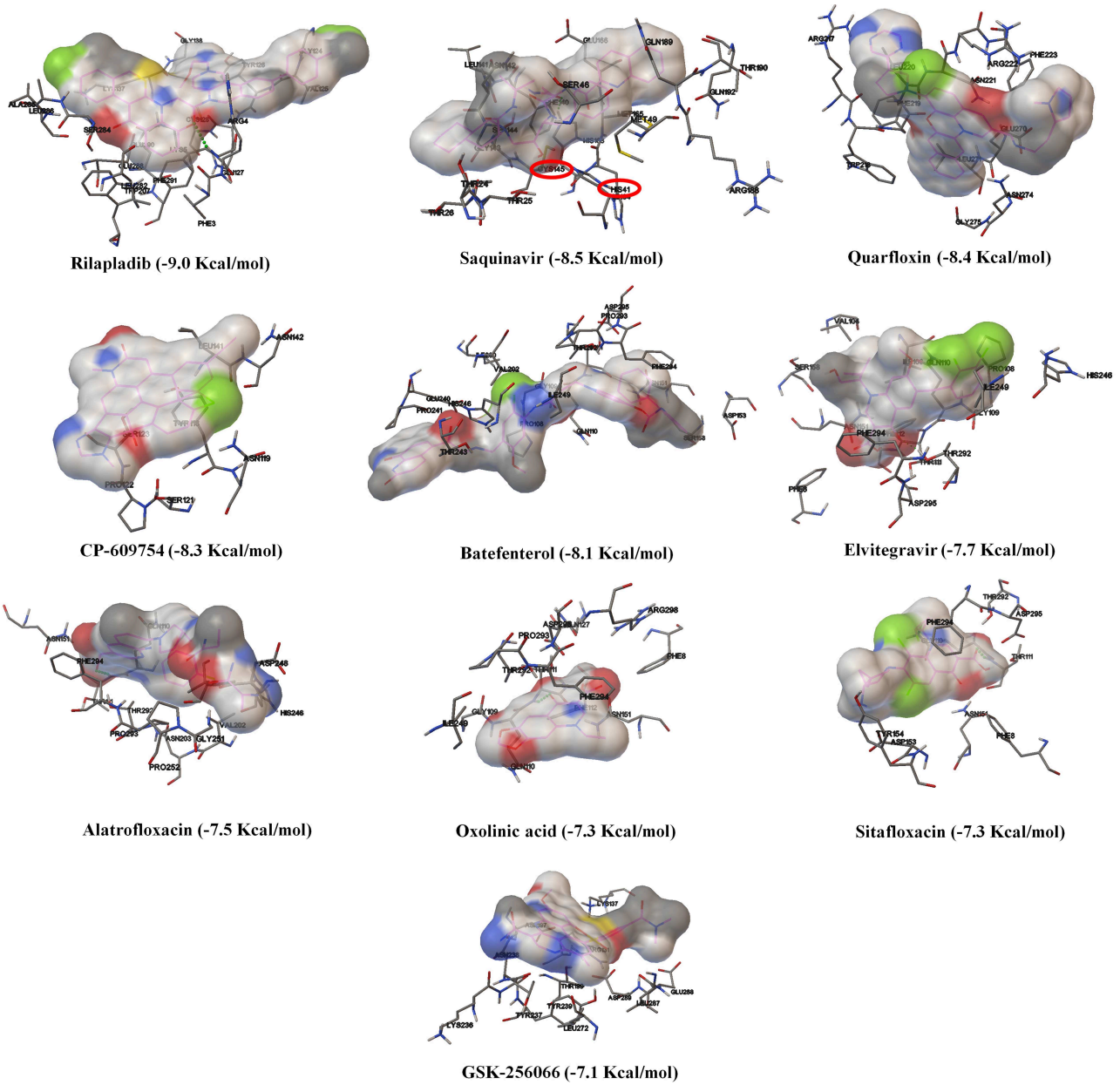


FIGURE 2 | The binding patterns and aminoacid interactions of the best scoring quinolines with SARS-CoV-2 3CLpro. The red circle-marking indicates the interaction with the active site of SARS-CoV-2 3CLpro (CysHis catalytic dyad).

and five unspecified residue interactions (Lys545, Arg553, Arg555, Lys621, and Lys798) with SARS-CoV-2 RdRp. Notably, elvitegravir builds a hydrogen bond interaction with Lys621 residue (1.931 Å distance), as shown in **Figure 4F**. Similarly, it formed five hydrophobic interactions (Tyr456, Met542, Ala554, Val557, and Ala558), six polar interactions (Thr556, Thr680, Ser681, Ser682, Thr687, and Asn691), two negative-charged interactions (Asp452 and Asp623), and five unspecified residue interactions (Lys545, Arg553, Arg555, Arg624, and Lys676) (**Figures 4D,J**). Subsequently, oxolinic acid forms three hydrogen bonding interactions with Thy456 (1.224Å), Ser682 (1.815Å), and Arg624 (2.904) residues of the SARS-CoV-2 RdRp (**Figure 4H**). As shown in **Figures 4E,G**, both elvitegravir and oxolinic acid have the ability to bind with the NTP binding channel (a set of hydrophilic residues such as Lys545, Arg553, and Arg555) of the SARS-CoV-2 RdRp, similar to remdesivir triphosphate (−7.8 Kcal/mol) (**Figure S3**), which is a RdRp-inhibitor based anti-SARS-CoV-2 agent reported for COVID-19 (Gao et al., 2020; Gordon et al., 2020). As shown in **Figure S4**, due to the same binding sites, we expect these two quinolines

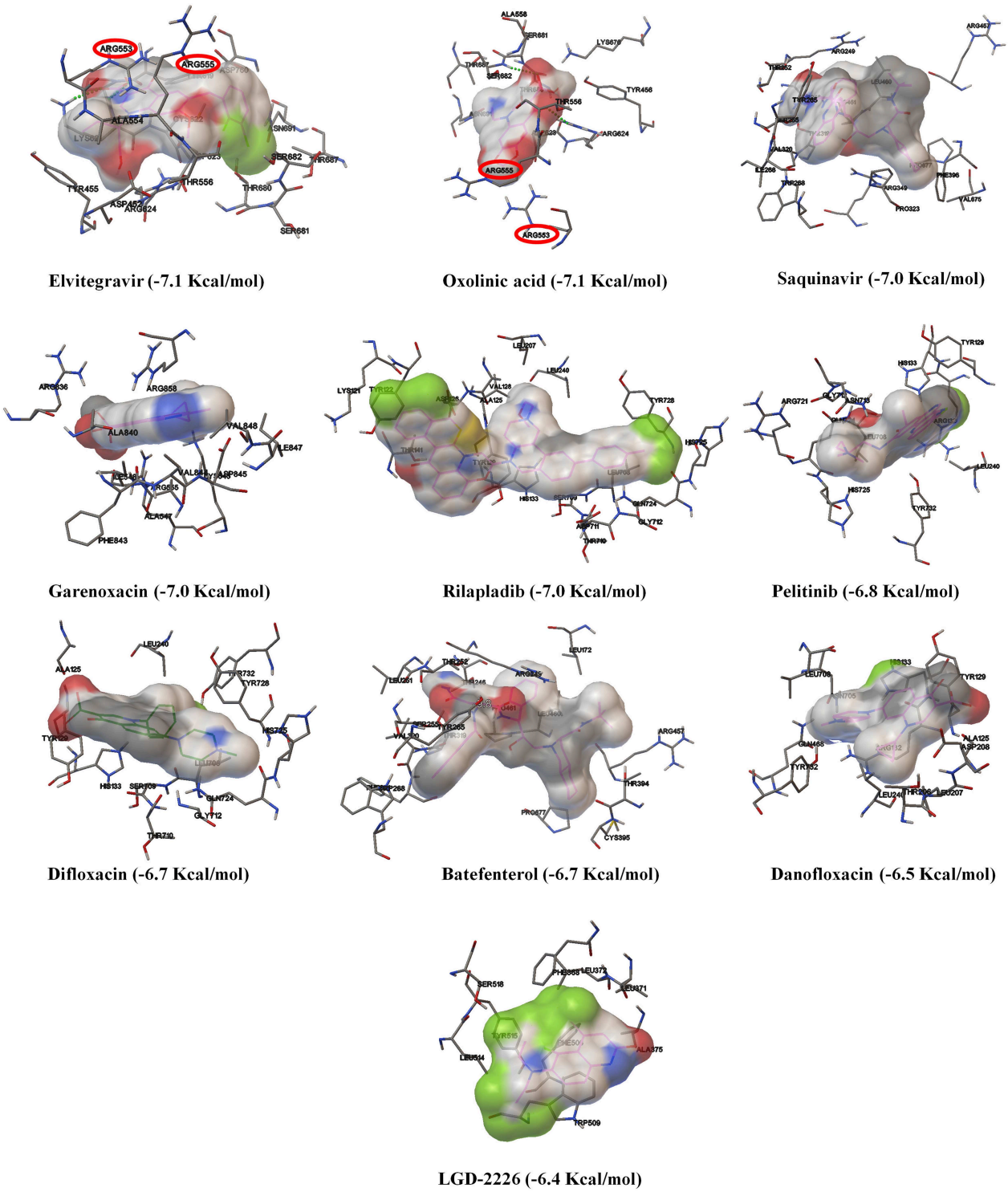


FIGURE 3 | The binding patterns and aminoacid interactions of the best scoring quinolines with SARS-CoV-2 RdRp. The red circle-marking indicates the interaction with the NTP entry channel of SARS-CoV-2.

(elvitegravir and oxolinic acid) can readily interact with the NTP binding channel of SARS-CoV-2 RdRp, more quickly than the parental-nucleotides (NTPs) such as ATP, UTP, GTP, and CTP. Hence, these two quinoline drugs are anticipated to arrest the viral replication of SARS-CoV-2, as is seen with remdesivir triphosphate.

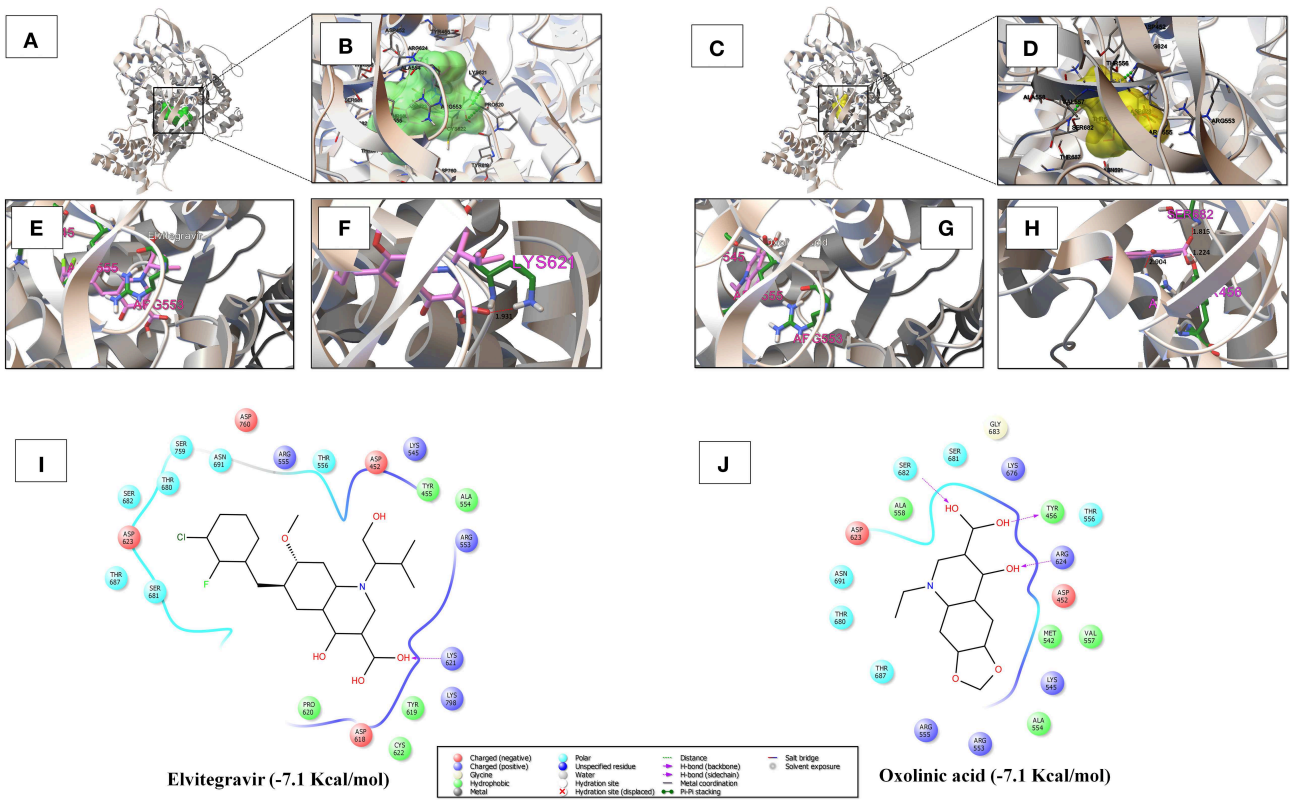


FIGURE 4 | (A,B) Shows the binding region and close-up view of the interacting amino acids of elvitegravir (−7.1 Kcal/mol) with SARS-CoV-2 RdRp, respectively. **(C,D)** Individually depicts the binding region and super-position view of the interacting amino acids of oxolinic acid (−7.1 Kcal/mol) with SARS-CoV-2 RdRp, respectively. **(E,G)** Visualizes the interaction with the NTP entry channel of SARS-CoV-2 RdRp (a set of hydrophilic residues such as Lys545, Arg553, and Arg555) of elvitegravir and oxolinic acid. **(F,H)** Shows the hydrogen bond formation of elvitegravir (Lys621 with a distance of 1.931 Å) and oxolinic acid (Thy456, Ser682, and Arg624 with a distance of 1.224, 1.815, and 2.904 Å, respectively). **(I,J)** Illustrate the interacted aminoacid residues of SARS-CoV-2 3CLpro with elvitegravir and oxolinic acid.

Screening of Potent Inhibitor for Spike Protein-RBD-ACE2 Interaction

In **Figure S5** and **Figure 6A**, the crystal structure of spike protein-ACE2 (PDB: 6M17) revealed that the aminoacid residues of ACE2, including Gln24, Thr27, Asp30, Lys31, His34, Glu35, Glu37, Asp38, Tyr41, Gln42, Met82, Lys353, Gly354, Asp355, and Arg357, were recognized as the Spike protein receptor-binding domain (RBD) or entry receptor site to invade the target cells (Benítez-Cardoza and Vique-Sánchez, 2020). Based on the virtual screening results of ACE2 protein, CP-609754, saquinavir, rilapladib, quarfloxin, batefenterol, oxolinic acid, alatrofloxacin, dovitinib, GSK-256066, and rebamipide were predicted as the best compounds, exhibiting a high binding affinity to ACE2 with low energy (**Figure 5** and **Table S4**). However, these quinolines were not predicted to interact with the RBD of the Spike-ACE2 complex (**Figure 5**). The only compound that could target the RBD interface between Spike and ACE2 was rilapladib, as shown in **Figure 6B**. Rilapladib was predicted to be positioned on the central shallow pit of the RBD of the Spike-ACE2 complex with strong interactions (**Figure 6D**). The aminoacid residues of the Spike-RBD-ACE2 complex that interact with rilapladib were His34, Glu35, Glu37, Asp38, and Leu39, as shown in **Figure 6C**. As a result of superimposing the Spike-RBD-ACE2 complex to the rilapladib-RBD-ACE2 complex, an individual overlap of rilapladib with the interface of ACE2 was observed, signifying that rilapladib may possibly interrupt the interaction of the Spike-RBD-ACE2 complex.

In silico Prediction of Drug-Likeness Property and Bioactivity Score

The adequacy of therapeutic drugs mainly depends on the molecular property and bioactivity of the compounds (Shen et al., 2012). To predict the drug-likeness and bioactivity of the selected quinolines, the *in silico* molecular property assessment was performed using the Molinspiration tool. This tool measures the $_{mi}\log P$ value (Octanol-water partition coefficient log*P*) and TPSA (Topological polar surface area) values of the compounds using Bayesian statistics. The result shows that the $_{mi}\log P$ value of quinoline,1,2,3,4-tetrahydro-1-[(2-phenylcyclopropyl)sulfonyl]-trans-(8CI) (3.63), saquinavir (4.26), elvitegravir (3.58), and oxolinic acid (0.68) were predicted as having ideal lipophilicity ($\log P < 5$) (Han et al., 2019); rilapladib (7.33) was predicted as having poor lipophilicity ($\log P > 5$) in the aspect of absorption and permeation

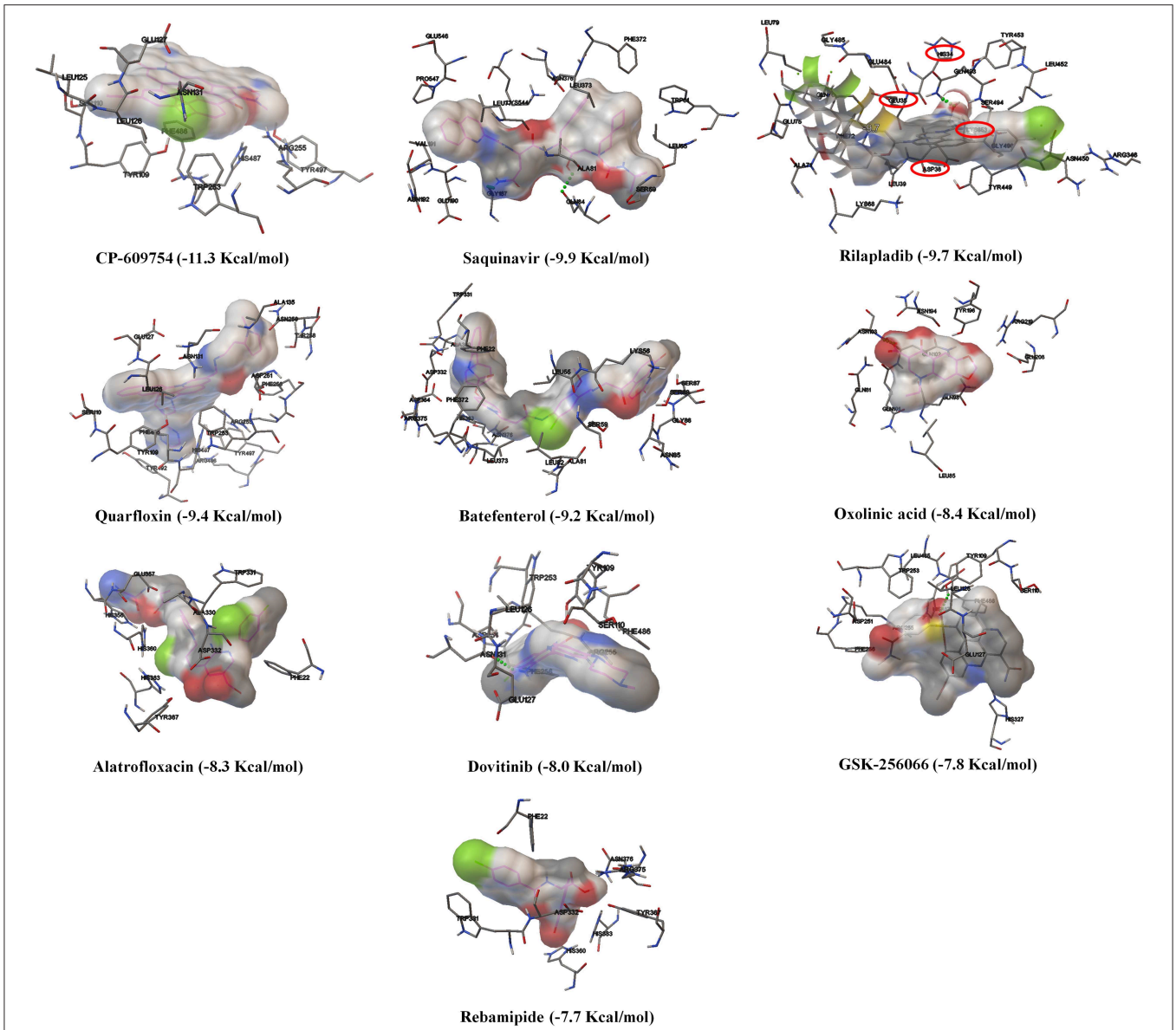


FIGURE 5 | The binding patterns and aminoacid interactions of the top ten scoring quinolines with the RBD interface of the Spike-ACE2 complex. The red circle-marking indicates the interaction with the RBD interface of Spike-ACE2 complex.

(**Figure S6**). The TPSA of the quinoline,1,2,3,4-tetrahydro-1-[(2-phenylcyclopropyl)sulfonyl]-trans-(8CI) (37.38), elvitegravir (88.77), oxolinic acid (77.77), and rilapladib (54.79) were <100, showing that these compounds had superior oral-absorption or membrane permeability than saquinavir (166.75), lopinavir (119.99), and remdesivir triphosphate (289.53) (Bakht et al., 2010). On the other hand, the majority of drug targets of existing drugs are in one of the following protein families: G protein-coupled receptors (GPCR), ion channels, kinases, nuclear hormone receptors, proteases, and other enzymes. In **Figure S7**, the *in silico* bioactivity prediction analysis also divulges that the NQ and saquinavir were predicted as protease inhibitors. Subsequently, elvitegravir and oxolinic acid were predicted as enzyme inhibitors, which means that these are able to inhibit other enzymes, including RdRp enyzme, except G protein-coupled receptors (GPCR), ion channels, kinases, nuclear hormone receptors, and proteases. This is because the protein families are the major drug targets of most of the drugs (Hauser et al., 2017). These data support the outcome of the predicted *in-silico* activity of selected quinolines against SARS-CoV-2.

The half-life ($t_{1/2}$) of drugs is a valuable pharmacokinetic factor as it provides an exact indication of the duration of time that the effect of the drug continues in an individual. The period of action of a drug is called its half-life. The distribution half-life ($t_{1/2a}$) is the time required to divide the plasma concentration

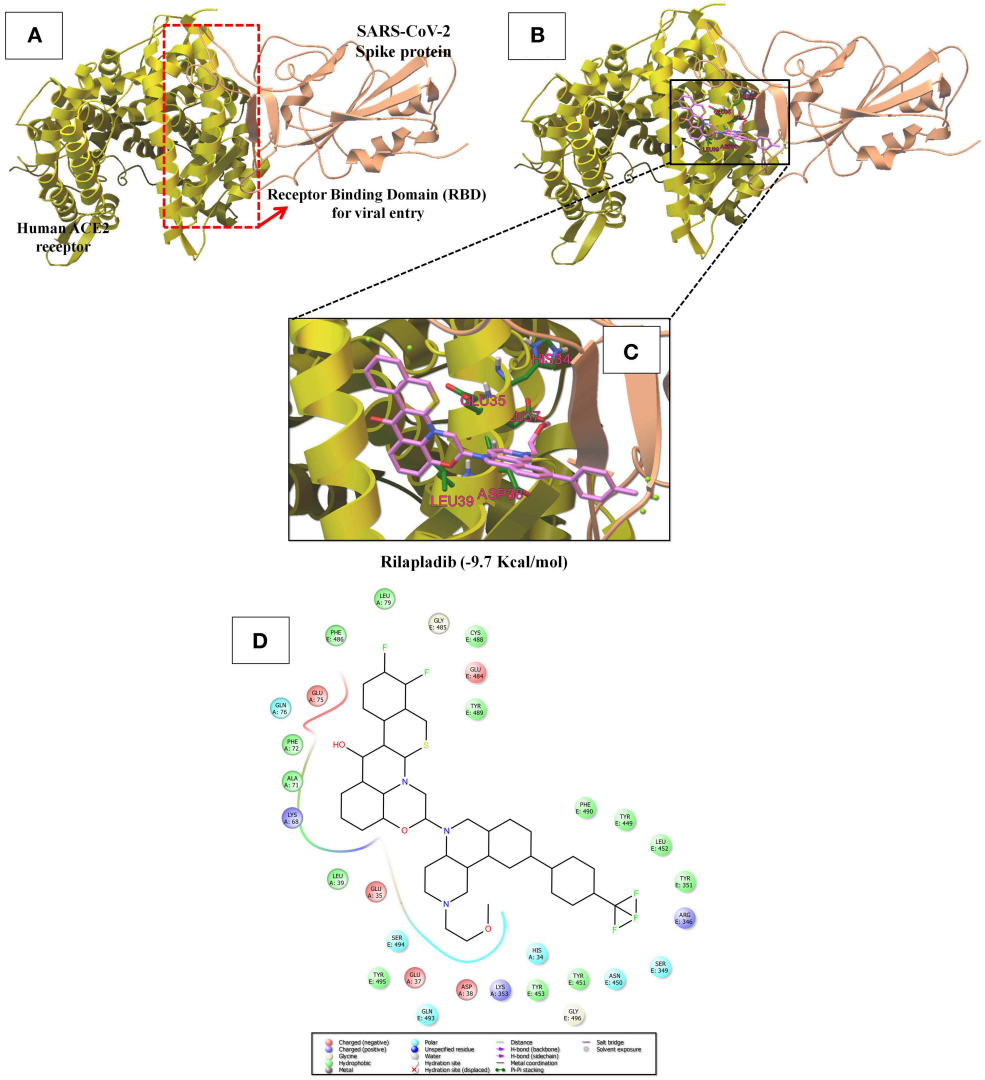
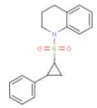
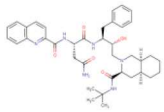
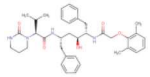
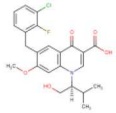
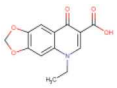
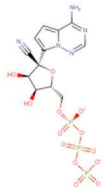
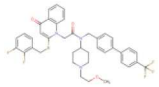


FIGURE 6 | (A) Shows the receptor binding domain (RBD) of SARS-CoV-2 Spike protein with human ACE-2 receptor for viral entry into host cells. **(B)** The interrupting mode of rilapladib (−9.7 Kcal/mol) on the interface of RBD of the Spike protein-ACE2 complex. **(C)** Super-position view of the binding interations of rilapladib on the interface of RBD of the Spike protein-ACE2 complex. **(D)** Shows the interacted aminoacid residues of rilapladib on the interface of RBD of the Spike protein-ACE2 complex.

by two after reaching pseudo-equilibrium, and not the time needed to eliminate half the administered dose. The elimination half-life ($t_{1/2b}$) of drugs is defined as the time required for the concentration of the drug to reach half of its original value in the plasma or the total amount in the body. As mentioned in **Table 1**, the elimination half-life ($t_{1/2b}$) of saquinavir (670.84 g/mol) is 7–12 h and their elimination half-life is 9–15 h (Taylor et al., 2001). The distribution half-life ($t_{1/2a}$) of elvitegravir is calculated at 8.7 h, and the elimination half-life ($t_{1/2b}$) as 12.9 h (Cada et al., 2013). Oxolinic acid (261.23 g/mol) has 1.3 and 84 h as its distribution half-life and elimination half-life, respectively (Samuelsen et al., 2003). Unfortunately, the half-life of rilapladib (735.81 g/mol) as well as quinoline,1,2,3,4-tetrahydro-1-[(2-phenylcyclopropyl)sulfonyl]-, trans-(8CI) (313.41 g/mol) are not available in the database. On the other hand, the elimination half-life of SARS-CoV-2 protease inhibitor lopinavir (628.8 g/mol) measured at 6.9 ± 2.2 h. Similarly, the reported anti-COVID-19 drug remdesivir has a very short half-life (0.39 h) and hence the human esterases hastily converted the remdesivir into nucleoside triphosphate metabolite (remdesivir triphosphate). However, the produced remdesivir triphosphate has a longer half-life of ∼20 h in humans, in which the metabolite acts as an NTP analog and slows down the viral replication of SARS-CoV-2 (Eastman et al., 2020). Due to the long half-life of the NTP analog of remdesivir triphosphate, only one dose is required for daily administration for treating COVID-19. The data obtained revealed that the selected quinolines are significantly long in terms of drug half-life, in which the quinolines treatment will be successful in the drug repurposing against COVID-19.

TABLE 1 | Half-life, molecular weight, and pharmacological functions of the suggested quinoline drugs for drug-repurposing against COVID-19.

S. No.	Drug/Compound Name	DrugBank ID	Structure	Molecular weight (g/mol)	Half-life (h)		Pharmacological function	Binding energy with the therapeutic targets of COVID-19	Drug-repurposing for COVID-19
					Distribution half-life ($t_{1/2a}$)	Elimination half-life ($t_{1/2b}$)			
1	Quinoline,1,2,3,4-tetrahydro-1-[(2-phenylcyclopropyl)sulfonyl]-, trans- (8Cl)	Not available		313.41	Not available	Not available	• Protease Inhibitor	−6.8 Kcal/mol with 3CLpro	In this study
2	Saquinavir	DB01232 (APRD00623)		670.84	7–12	9–15	• Anti-HIV Agent (Kim et al., 1998) • Anti-Infective Agent (Noble and Faulds, 1996) • Protease Inhibitor (Kim et al., 1998)	−8.5 Kcal/mol with 3CLpro	In this study
3	Lopinavir (Reported anti-SARS-CoV-2 agent)	DB01601 (EXPT00388)		628.8	Not available	6.9 ± 2.2	• Experimental Unapproved Treatment for COVID-19 (Choy et al., 2020) • Anti-HIV Agent (Walmsley et al., 2002) • Protease Inhibitor (Agarwal et al., 2007)	−6.6 Kcal/mol with 3CLpro	Choy et al., 2020
4	Elvitegravir	DB09101 (DB05618)		447.88	8.7	12.9	• Anti-HIV Agent (Ramanathan et al., 2011) • Anti-viral for Systemic Use (Lampiris, 2012) • Enzyme Inhibitor (Shimura et al., 2008)	−7.1 Kcal/mol with RdRp	In this study
5	Oxolinic acid	DB13627		261.23	1.3	84	• Anti-bacterial Agent (Barry et al., 1984) • Anti-Infective Agent for Urinary Infections (Irgi et al., 2015) • Enzyme Inhibitor (Wright et al., 1981)	−7.1 Kcal/mol with RdRp	In this study
6	Remdesivir triphosphate (Reported anti-SARS-CoV-2 agent)	DB14761		527.17	Not available	20	• Experimental Unapproved Treatment for COVID-19 (Gordon et al., 2020; Wang Y. et al., 2020)	−7.8 Kcal/mol with RdRp	Gordon et al., 2020; Wang Y. et al., 2020
7	Rilapladib	DB05119 (DB05256)		735.81	Not available	Not available	• Anti-Alzheimer's Disease (Husna Ibrahim et al., 2020) • Lp-PLA2 inhibitor (Shaddinger et al., 2014)	−9.7 Kcal/mol with Spike protein-ACE2 complex	In this study

In silico ADMET Analysis

In **Table 2**, the absorption (A) analysis reveals that the NQ and oxolinic acid were predicted to have high Caco-2 permeability. A HIA of <30% is classified to be poorly absorbed. The result reveals that all the quinolines were predicted to be highly absorbed by the human intestine. P-glycoprotein, a member of ATP-binding trans-membrane glycoprotein, excretes incoming drugs or other chemicals from the cells (Äänismaa and Seelig, 2007). The results revealed that the NQ and oxolinic acid are non-substrates or non-inhibitors for P-glycoprotein and other compounds are substrates/inhibitors for P-glycoprotein. In the distribution (D) analysis, the BBB permeability, logBBB >0.3, is thought to cross the BBB easily (Han et al., 2019). Here, all the quinolines were predicted to be BBB+ and they can easily cross the BBB. In the metabolism (M) analysis, the two main sub-types of cytochrome P450s (CYP) are CYP2D6 and CYP3A4, which are essential enzyme-systems for drug metabolism in the liver (Sams et al., 2004). The results showed that all the quinolines were non-inhibitors to CYP2D6; only NQ and saquinavir were substrates for CYP2D6 and CYP3A4. This data suggested that these quinolines may possibly metabolize in the liver. OATP1B1 and OATP1B3 are transporters expressed on the sinusoidal-membrane of hepatocytes, which interact with therapeutic-drugs as their substrates or inhibitors, and cause clinically relevant drug-drug interactions (Shitara, 2010). The result found that all the selected quinolines were predicted as inhibitors for OATP1B1-OATP1B3. The excretion (E) of drugs is associated with their hydrophilicity and molecular weight. In the kidney, organic cation transporters (OCTs) and multidrug and toxin extrusion proteins (MATEs) are the foremost transporters for the clearance of cationic drugs into the urine (Motohashi and Inui, 2013). The results showed that the quinolines were predicted as non-inhibitors to MATE-1, OCT-1, and OCT-2, which indicates the safety elimination profile of the quinolines. In the toxicity (T) analysis, the result showed that all quinolines were non-carcinogen and non-eye corrosive. In conclusion, the predicted results indicate that the ADMET properties of the quinolines are almost similar to the reported anti-SARS-CoV-2 drugs (lopinavir and remdesivir triphosphate), which promotes repurposing of these quinoline drugs for the treatment of COVID-19.

DISCUSSION

As significant functional biological-macromolecules of coronavirus, the viral protease (3CLpro) and RNA-dependent

TABLE 2 | Predicted ADMET properties of the selected quinolines.

ADME parameters	Novel quinoline	Saquinavir	Elvitegravir	Oxolinic acid	Rilapladib	Lopinavir	Remdesivir triphosphate
Absorption							
Caco-2 permeability	+	–	–	+	+	+	–
Human Intestinal Absorption (% absorbed)	+ 88.96%	+ 98.13%	+ 96.44%	+ 93.75%	+ 92.54%	+ 96.24%	+ 93.17%
P-glycoprotein inhibitor	–	+	+	–	+	+	+
P-glycoprotein substrate	–	+	+	–	+	+	+
Distribution							
Blood Brain Barrier	+	+	+	+	+	+	+
Subcellular localization	Plasma membrane	Mitochondria	Lysosomes	Mitochondria	Mitochondria	Mitochondria	Lysosomes
Metabolism							
CYP2D6 inhibition	–	–	–	–	–	–	–
CYP2D6 substrate	+	+	–	–	–	–	–
CYP3A4 inhibition	+	+	–	–	–	–	–
CYP3A4 substrate	–	+	+	–	+	+	+
OATP1B1 inhibitor	+	+	+	+	+	+	+
OATP1B3 inhibitor	+	+	+	+	+	+	+
Excretion							
OCT1 inhibitor	–	–	–	–	–	–	–
OCT2 inhibitor	–	–	–	–	–	–	–
MATE1 inhibitor	–	–	–	–	–	–	–
Toxicity							
Carcinogens	–	–	–	–	–	–	–
Acute-toxicity (Class)	III	III	III	III	III	III	III
Eye corrosion	–	–	–	–	–	–	–
Eye irritation	–	–	–	+	–	–	–
Human either-a-go-go inhibition	+	+	–	–	+	+	–

The predicted properties are color-coded to enable easy classification among the quinolines. The color codes are: red for toxic or inhibitor, orange for weak inhibitor or slightly toxic, green for safe or non-inhibitor. The symbols include + for yes and – for no.

RNA-polymerase (RdRp) are indispensable for proteolytic processing of the polyproteins as well as viral replication and have been considered as promising drug targets in the treatment of viral diseases (Zumla et al., 2016). Several drugs, including hydroxychloroquine, chloroquine, arbidol, remdesivir, favipiravir, lopinavir/ritonavir, interferon-α, and ribavirin are undergoing clinical trials to assess their anti-viral efficacy and safety level in the treatment of COVID-19 (Dong et al., 2020). Most of the reported anti-SARS-CoV-2 drugs are protease-inhibitors or RdRp-inhibitors (Elfiky, 2020; Wu et al., 2020).

One of the most-characterized therapeutic targets among coronaviruses is inhibiting the 3CLpro activity since this enzyme is crucial for processing the polyproteins that are translated from the RNA molecules (Ghosh et al., 2007; Khan et al., 2020). The 3CLpro, also called Nsp5 (non-structural protein 5), is first routinely cleaved from polyproteins to produce mature enzymes, and subsequently further cleaves downstream Nsps at 11 cleavage sites to release Nsp4-Nsp16 (Wu et al., 2020). 3CLpro directly mediates the maturation of Nsps, which is fundamental in the life-cycle of SARS-CoV-2 (Zhang H. et al., 2020). The 3CLpro monomer has three domains, namely domain I (residues 8–101), domain II (residues 102–184), and domain III (residues 201–303), and a long loop (residues 185–200) links domains II and III, as shown in **Figure 1A**. The active site of 3CLpro is located in the gap between domains I and II, and has a CysHis catalytic dyad (Cys145 and His41) (Jin et al., 2019; Wu et al., 2020).The active site of SARS-CoV-2 3CLpro is located in the gap between domains I and II, and has a CysHis catalytic dyad (Cys145 and His41) (Muralidharan et al., 2020). The cleavage by 3CLpro arises at the glutamine residue in the P1 position of the substrate through the CysHis catalytic dyad, wherein cysteine thiol functions as the nucleophile in the proteolytic process (Chen et al., 2005). Hence, inhibiting the activity of this enzyme would arrest the viral replication of SARS-CoV-2.

We saw that quinoline,1,2,3,4-tetrahydro-1-[(2-phenylcyclopropyl)sulfonyl]-trans-(8CI) (NQ) and saquinavir can target main proteases through authoritative interaction to the catalytic dyad (Cys145 and His41) of SARS-CoV-2 3CLpro (**Figures 1F,H**), and along these lines are believed to hinder the protease activity, as has been reported with anti-SARS-CoV-2 agent (lopinavir). As referenced before, we identified the NQ from the methanolic leaf extract of *D. palmatus* using GC-MS analysis (Alexpandi et al., 2019). Unfortunately, the NQ is not available commercially. Henceforth, the present study essentially evaluated their drug-likeness, bioactivity, and ADMET properties through an *in silico* approach. The outcomes demonstrated that the NQ has low $_{mi}\log P$ (3.63) and low TPSA value (37.38), which authenticates the ideal lipophilicity ($_{mi}\log P < 5$) nature and higher oral-absorption or membrane permeability (TPSA < 100) than other quinolines. These are the physicochemical properties that play a fundamental role in deciding the ADMET properties of compounds (Shen et al., 2012; Han et al., 2019). In ADMET analysis, the results reveal that the NQ was predicted as Caco-2+, HIA+, a non-inhibitor to P-glycoprotein, BBB+, a non-inhibitor to CYP2D6, a non-substrate to CYP3A4, and a non-inhibitor to excretion-related receptors such as OCT-1, OCT-2, and MATE-1. The NQ showing some toxic impact on the human either-a-go-go related genes, has even been predicted as a non-carcinogenic, non-mutagenic, non-eye irritant, and non-eye corrosive. These results unequivocally suggest that the novel phytocompound, quinoline,1,2,3,4-tetrahydro-1-[(2-phenylcyclopropyl)sulfonyl]-trans-(8CI), can be used as a protease-inhibitor drug for the treatment of COVID-19, but the compound needs to be synthesized.

Saquinavir, the first FDA-approved HIV-1 drug, has the ability to cleave between Tyr-Pro or Phe-Pro of the HIV polyproteins, which is rare in mammalian systems (Noble and Faulds, 1996). Hence, saquinavir does not interfere with mammalian proteases, symbolizing its safety level for humans (Ganguly et al., 2011). Saquinavir is a protease inhibitor that binds to the active site of the viral protease and thereby blocks cleavage of viral polyproteins and maturation of the HIV-1 and HIV-2 (Kim et al., 1998). Furthermore, (Tan et al., 2004) showed the *in vitro* antiviral activity of saquinavir against SARS-CoV-1. The present study revealed that saquinavir is able to bind with the catalytic dyad, and is thereby anticipated to interrupt 3CLpro activity (**Figure 1H**). So, we recommend saquinavir as the potent protease-inhibitor for drug-repurposing against COVID-19.

In the research of anti-SARS-CoV-2 drug designing, RdRp has been well-thought-out as an incredibly potent drug target due to its central role in RNA-synthesis from RNA-templates (Gao et al., 2020). In addition, RdRp-inhibitors do not show considerable toxicity or side effects on host cells (Dong et al., 2020). The active site of the SARS-CoV-2 RdRp domain is formed by the conserved polymerase motifs A-G, within 549th to 776th aminoacid residues, which are essential for the RNA-directed 5'-3' polymerase activity (Shannon et al., 2020). As in other RNA-polymerases, the template/primer entry (known as nucleoside triphosphate (NTP) entry) and budding strand are congregating in a central cavity where the RdRp motifs intercede RNA-template mediated RNA synthesis in SARS-CoV-2. The NTP entry channel of SARS-CoV-2 is placed in the set of hydrophilic residues, including Lys545, Arg553, and Arg555 in motif-F (Gao et al., 2020). Therefore, the nucleotide-analog antiviral inhibitors such as remdesivir and favipiravir also showed their antiviral potential against SARS-CoV-2 (Gordon et al., 2020). Our results revealed that both elvitegravir and oxolinic acid have the ability to bind with the NTP binding channel of SARS-CoV-2 with a low binding energy (−7.1 Kcal/mol) (**Figures 4E,G**), similar to the parental nucleotides such as ATP (−7.6 Kcal/mol), UTP (−7.1 Kcal/mol), GTP (−7.7 Kcal/mol), and CTP (−7.1 Kcal/mol). We believed that these two quinolines can more readily interact with the NTP binding channel than parental-nucleotides (especially than UTP and CTP), and thereby, possibly block the *de novo* addition of NTP to the 3'-OH strand, which leads to the arrest of viral replication (Gao et al., 2020), as illustrated in **Figure S4**.

Elvitegravir is an integrase inhibitor used for the anti-retroviral treatment of HIV-1 (Shimura et al., 2008; Ramanathan et al., 2011). Oxolinic acid is a synthetic quinoline-derived antibiotic used to treat bacteria causing urinary tract infections (Sato et al., 2006; Irgi et al., 2015). Owing to the nucleotide-antagonistic behavior, we suggest that elvitegravir and oxolinic acid might be the potent RdRp inhibitors for SARS-CoV-2 in the treatment of COVID-19.

On the other hand, ACE2 is a type I transmembrane metallocarboxypeptidase, an enzyme that plays a crucial role in the rennin-angiotensin (RAS) system and is considered as a target for the treatment of hypertension (Burrell et al., 2004). ACE2 is widely distributed in the human body and has been associated with the protective function in the cardiovascular system and other organs (Yagil et al., 2003). In contrast, human ACE2 is the recognized functional receptor for the Spike glycoprotein of SARS-CoV-2 that initiates cell entry into host cells and viral replication in the target cells (Lan et al., 2020). The previous report proved that ACE2 knockout significantly reduces the viral load in mice after the experimental SARS-CoV infection (Hoffmann et al., 2020). As shown in **Figure 3**, though several quinolines could bind with ACE2, none was found to bind with the RBD of the ACE2-Spike complex. Moreover, these kinds of ACE2 inhibitors may not be appropriate for treating COVID-19 because these drugs can inhibit ACE2 enzyme activities, and cause lung injury and heart failure (Velkoska et al., 2016). To predict the RBD interface binding compound, we found one quinoline-drug, rilapladib, was targeting the RBD of the Spike-ACE2 complex, as shown in **Figure 6B**. Rilapladib, a hydroquinoline-based small molecule drug developed by GlaxoSmithKline was used as a lipoprotein-associated phospholipase A2 (Lp-PLA2) inhibitor or 1-alkyl-2-acetylglycerophosphocholine esterase inhibitor for treating atherosclerotic plaques and Alzheimer's disease (Shaddinger et al., 2014). It's worth mentioning that rilapladib was well-fitted into the interface of the RBD of the Spike-ACE2 complex (**Figure 6B**), in which lots of interactions with His34, Glu35, Glu37, Asp38, Leu39, Lys68, Ala71, Phe72, Glu75, Gln76, Leu79, and Lys353 create a strong binding with the interface of RBD and block the Spike-ACE2 interactions. Owing to the formation of possible interactions at the RBD interface of the Spike-ACE2 complex, the present study suggests rilapladib prevents ACE2-mediated viral entry of SARS-CoV-2.

Moreover, the *in silico* ADMET results demonstrated that these quinolines were non-toxic, non-carcinogenic, absorb in the human intestine, have Caco-2 permeability, do not inhibit CYP enzymes, are non-inhibitors for RCT, and non-inhibitors for Human Ether-a-go-go related genes, which suggested their significant pharmacokinetic properties. Further, the drug half-life of selected quinoline drugs are significantly long, in which these quinolines were expected to offer an efficient drug distribution against COVID-19. Overall, we believe that these quinolines may be efficient drug candidates for the development of efficient therapeutics against COVID-19 in this pandemic period.

CONCLUSION

In conclusion, the current investigation recommends that the novel phyto-quinoline (quinoline,1,2,3,4-tetrahydro-1-[(2-phenylcyclopropyl)sulfonyl]-trans-(8CI)) and the existing quinoline-based drugs saquinavir, elvitegravir, and oxolinic acid could be used as potent inhibitors for SARS-CoV-2. Subsequently, rilapladib is suggested for anti-Spike-RBD-ACE2 therapy to avoid ACE2-mediated viral entry of SARS-CoV-2 into the host cells. Notwithstanding, further *in vitro* and *in vivo* experiments are needed to transform these potential inhibitors into clinical drugs. We anticipate that this new finding could significantly impact the development of therapeutic agents for COVID-19 in the future.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

RA: conceptualization, performed the experiments, data analysis, and writing-original draft. JD: data analysis and reviewing-original draft. SP: supervision and reviewing-original draft. AR: conceptualization, supervision, and reviewing-original draft. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

RA sincerely acknowledges Rashtriya Uchchatar Shiksha Abhiyan (RUSA), MHRD, Government of India [F. 24-51/2014-U, Policy (TN Multi-Gen), Dept. of Edn, GOI] for providing RUSA-2.0 Ph.D. Fellowship. We sincerely thank Prof. Hua Li, Huazhong University of Science and Technology, Wuhan, China for providing the modeled-structure of SARS-CoV-2 RdRp. RA thanks Mr. Ragupathi Thennarasu, DBT-BIF Research Assistant, ALU for assistance during data analysis. We also acknowledge the BIF (funded by DBT, File No. BT/BI/25/012/2012), DST-FIST [Grant No. SR/FST/LSI-639/2015(C)], UGC-SAP [Grant No. F.5-1/2018/DRS-II (SAP-II)], DST-PURSE [Grant No. SR/PURSE Phase 2/38 (G)], and RUSA- Phase 2.0, Government of India [F. 24-51/2014-U, Policy (TN Multi-Gen)] for providing instrumentation facilities.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.01796/full#supplementary-material

REFERENCES

Äänismaa, P., and Seelig, A. (2007). P-Glycoprotein kinetics measured in plasma membrane vesicles and living cells. *Biochemistry* 46, 3394–3404. doi: 10.1021/bi0619526

Agarwal, S., Pal, D., and Mitra, A. K. (2007). Both P-gp and MRP2 mediate transport of Lopinavir, a protease inhibitor. *Int. J. Pharm.* 339, 139–147. doi: 10.1016/j.ijpharm.2007.02.036

Alexpandi, R., Prasanth, M. I., Ravi, A. V., Balamurugan, K., Durgadevi, R., Srinivasan, R., et al. (2019). Protective effect of neglected plant Diplocyclos palmatus on quorum sensing mediated infection of *Serratia marcescens* and UV-A induced photoaging in model *Caenorhabditis elegans*. *J. Photoch. Photobio. B* 201:111637. doi: 10.1016/j.jphotobiol.2019.111637

Bakht, M. A., Yar, M. S., Abdel-Hamid, S. G., Al Qasoumi, S. I., and Samad, A. (2010). Molecular properties prediction, synthesis and antimicrobial activity of some newer oxadiazole derivatives. *Eur. J. Med. Chem.* 45, 5862–5869. doi: 10.1016/j.ejmech.2010.07.069

Balasubramaniam, B., Alexpandi, R., and Darjily, D. R. (2019). Exploration of the optimized parameters for bioactive prodigiosin mass production and its biomedical applications *in vitro* as well as *in silico*. *Biocatal. Agric. Biotechnol.* 22:101385. doi: 10.1016/j.bcab.2019.101385

Barry, A. L., Jones, R. N., Thornsberry, C., Ayers, L. W., Gerlach, E. H., and Sommers, H. M. (1984). Antibacterial activities of ciprofloxacin, norfloxacin, oxolinic acid, cinoxacin, and nalidixic acid. *Antimicrob. Agents. Ch.* 25, 633–637.

Beetge, E., du Plessis, J., Müller, D. G., Goosen, C., and van Rensburg, F. J. (2000). The influence of the physicochemical characteristics and pharmacokinetic properties of selected NSAID's on their transdermal absorption. *Int. J. Pharm.* 193, 261–264. doi: 10.1016/S0378-5173(99)00340-3

Benítez-Cardoza, C. G., and Vique-Sánchez, J. L. (2020). Potential inhibitors of the interaction between ACE2 and SARS-CoV-2 (RBD), to develop a drug. *Life. Sci.* 256:117970. doi: 10.1016/j.lfs.2020.117970

Burrell, L. M., Johnston, C. I., Tikellis, C., and Cooper, M. E. (2004). ACE2, a new regulator of the renin–angiotensin system. *Trends. Endocrin. Met.* 15, 166–169. doi: 10.1016/j.tem.2004.03.001

Cada, D. J., Torres, S., Levien, T. L., and Baker, D. E. (2013). Elvitegravir/Cobicistat/Emtricitabine/Tenofovir disoproxil fumarate tablets. *Hosp. Pharm.* 48, 48–56. doi: 10.1310/hpj4801-48

Chen, L. R., Wang, Y. C., Lin, Y. W., Chou, S. Y., Chen, S. F., Liu, L. T., et al. (2005). Synthesis and evaluation of isatin derivatives as effective SARS coronavirus 3CL protease inhibitors. *Bioorg. Med. Chem. Lett.* 15, 3058–3062. doi: 10.1016/j.bmcl.2005.04.027

Choy, K. T., Wong, A. Y. L., Kaewpreedee, P., Sia, S. F., Chen, D., Hui, K. P. Y., et al. (2020). Remdesivir, lopinavir, emetine, and homoharringtonine inhibit SARS-CoV-2 replication *in vitro*. *Antivir. Res.* 178:104786. doi: 10.1016/j.antiviral.2020.104786

Dey, S. K., Rahman, M. M., Siddiqi, U. R., and Howlader, A. (2020). Analyzing the epidemiological outbreak of COVID-19: a visual exploratory data analysis approach. *J. Med. Virol.* 92, 632–638. doi: 10.1002/jmv.25743

Dong, L., Hu, S., and Gao, J. (2020). Discovering drugs to treat coronavirus disease 2019 (COVID-19). *Drug. Discov. Ther.* 14, 58–60. doi: 10.5582/ddt.2020.01012

Eastman, R. T., Roth, J. S., Brimacombe, K. R., Simeonov, A., Shen, M., Patnaik, S., et al. (2020). Remdesivir: a review of its discovery and development leading to emergency use authorization for treatment of COVID-19. *ACS Cent. Sci.* 6, 672–683. doi: 10.1021/acscentsci.0c00489

Elfiky, A. A. (2020). Ribavirin, Remdesivir, Sofosbuvir, Galidesivir, and Tenofovir against SARS-CoV-2 RNA dependent RNA polymerase (RdRp): a molecular docking study. *Life. Sci.* 253:117592. doi: 10.1016/j.lfs.2020.117592

Ganguly, A. K., Alluri, S. S., Caroccia, D., Biswas, D., Wang, C. H., Kang, E., et al. (2011). Design, synthesis, and X-ray crystallographic analysis of a novel class of HIV-1 protease inhibitors. *J. Med. Chem.* 54, 7176–7183. doi: 10.1021/jm200778q

Gao, Y., Yan, L., Huang, Y., Liu, F., Zhao, Y., Cao, L., et al. (2020). Structure of the RNA-dependent RNA polymerase from COVID-19 virus. *Science* 368, 779–782. doi: 10.1126/science.abb7498

Ghosh, A. K., Xi, K., Grum-Tokars, V., Xu, X., Ratia, K., Fu, W., et al. (2007). Structure-based design, synthesis, and biological evaluation of peptidomimetic SARS-CoV 3CLpro inhibitors. *Bioorg. Med. Chem. Lett.* 17, 5876–5880. doi: 10.1016/j.bmcl.2007.08.031

Goodsell, D. S., Morris, G. M., and Olson, A. J. (1996). Automated docking of flexible ligands: applications of AutoDock. *J. Mol. Recognit.* 9, 1–5.

Gordon, C. J., Tchesnokov, E. P., Woolner, E., Perry, J. K., Feng, J. Y., Porter, D. P., et al. (2020). Remdesivir is a direct-acting antiviral that inhibits RNA-dependent RNA polymerase from severe acute respiratory syndrome coronavirus 2 with high potency. *J. Biol. Chem.* 295:jbc.RA120.013679. doi: 10.1074/jbc.RA120.013679

Guan, L., Yang, H., Cai, Y., Sun, L., Di, P., Li, W., et al. (2019). ADMET-score–a comprehensive scoring function for evaluation of chemical drug-likeness. *Medchemcomm* 10, 148–157. doi: 10.1039/C8MD00472B

Hall D. C. Jr., and Ji, H. F. (2020). A search for medications to treat COVID-19 via *in silico* molecular docking models of the SARS-CoV-2 spike glycoprotein and 3CL protease. *Travel. Med. Infect. Dis.* 35:101646. doi: 10.1016/j.tmaid.2020.101646

Han, Y., Zhang, X., Zhang, J., and Hu, C. Q. (2019). *In silico* ADME and toxicity prediction of ceftazidime and its impurities. *Front. Pharmacol.* 10:434. doi: 10.3389/fphar.2019.00434

Hauser, A. S., Attwood, M. M., Rask-Andersen, M., Schiöth, H. B., and Gloriam, D. E. (2017). Trends in GPCR drug discovery: new agents, targets and indications. *Nat. Rev. Drug Discov.* 16, 829–842. doi: 10.1038/nrd.2017.178

Hoffmann, M., Kleine-Weber, H., Schroeder, S., Krüger, N., Herrler, T., Erichsen, S., et al. (2020). SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor. *Cell* 181, 271–280.e8. doi: 10.1016/j.cell.2020.02.052

Hsu, K. C., Chen, Y. F., Lin, S. R., and Yang, J. M. (2011). iGEMDOCK: a graphical environment of enhancing GEMDOCK using pharmacological interactions and post-screening analysis. *BMC bioinformatics* 12:S33. doi: 10.1186/1471-2105-12-S1-S33

Husna Ibrahim, N., Yahaya, M. F., Mohamed, W., Teoh, S. L., Hui, C. K., and Kumar, J. (2020). Pharmacotherapy of Alzheimer's Disease: seeking clarity in a time of uncertainty. *Front. Pharmacol.* 11:261. doi: 10.3389/fphar.2020.00261

Hussaini, S. M. A. (2016). Therapeutic significance of quinolines: a patent review (2013-2015). *Expert. Opin. Ther. Pat.* 26, 1201–1221. doi: 10.1080/13543776.2016.1216545

Irgi, E. P., Geromichalos, G. D., Balala, S., Kljun, J., Kalogiannis, S., Papadopoulos, A., et al. (2015). Cobalt (II) complexes with the quinolone antimicrobial drug oxolinic acid: structure and biological perspectives. *RSC Adv.* 5, 36353–36367. doi: 10.1039/C5RA05308K

Jin, Z., Du, X., Xu, Y., Deng, Y., Liu, M., Zhao, Y., et al. (2019). Structure of M pro from SARS-CoV-2 and discovery of its inhibitors. *Nature* 582, 289–293. doi: 10.1038/s41586-020-2223-y

Joshi, T., Joshi, T., Sharma, P., Mathpal, S., Pundir, H., Bhatt, V., et al. (2020). *In silico* screening of natural compounds against COVID-19 by targeting Mpro and ACE2 using molecular docking. *Eur. Rev. Med. Pharmacol. Sci.* 24, 4529–4536. doi: 10.26355/eurrev_202004_21036

Kaur, K., Jain, M., Reddy, R. P., and Jain, R. (2010). Quinolines and structurally related heterocycles as antimalarials. *Eur. J. Med. Chem.* 45, 3245–3264. doi: 10.1016/j.ejmech.2010.04.011

Khan, R. J., Jha, R. K., Amera, G., Jain, M., Singh, E., Pathak, A., et al. (2020). Targeting SARS-Cov-2: a systematic drug repurposing approach to identify promising inhibitors against 3C-like Proteinase and 2'-O-RiboseMethyltransferase. *J. Biomol. Struct. Dyn.* 1–40. doi: 10.1080/07391102.2020.1753577. [Epub ahead of print].

Kim, A. E., Dintaman, J. M., Waddell, D. S., and Silverman, J. A. (1998). Saquinavir, an HIV protease inhibitor, is transported by P-glycoprotein. *J. Pharmacol. Exp. Ther.* 286, 1439–1445.

Lampiris, H. W. (2012). Elvitegravir: a once-daily, boosted, HIV-1 integrase inhibitor. *Expert. Rev. Anti-infe.* 10, 13–20. doi: 10.1586/eri.11.157

Lan, J., Ge, J., Yu, J., Shan, S., Zhou, H., Fan, S., et al. (2020). Structure of the SARS-CoV-2 spike receptor-binding domain bound to the ACE2 receptor. *Nature* 581, 215–220. doi: 10.1038/s41586-020-2180-5

Mabkhot, Y. N., Alatibi, F., El-Sayed, N. N., Al-Showiman, S., Kheder, N. A., Wadood, A., et al. (2016). Antimicrobial activity of some novel armed thiophene derivatives and petra/osiris/molinspiration (POM) analyses. *Molecules* 21:222. doi: 10.3390/molecules21020222

Motohashi, H., and Inui, K. I. (2013). Organic cation transporter OCTs (SLC22) and MATEs (SLC47) in the human kidney. *AAPS J.* 15, 581–588. doi: 10.1208/s12248-013-9465-7

Muralidharan, N., Sakthivel, R., Velmurugan, D., and Gromiha, M. M. (2020). Computational studies of drug repurposing and synergism of lopinavir, oseltamivir and ritonavir binding with SARS-CoV-2 Protease against COVID-19. *J. Biomol. Struct. Dyn.* 1–6. doi: 10.1080/07391102.2020.1752802. [Epub ahead of print].

Noble, S., and Faulds, D. (1996). Saquinavir. *Drugs.* 52, 93–112. doi: 10.2165/00003495-199652010-00007

O'donnell, F., Smyth, T. J. P., Ramachandran, V. N., and Smyth, W. F. (2010). A study of the antimicrobial activity of selected synthetic and naturally occurring quinolines. *Int. J. Antimicrob. Agents.* 35, 30–38. doi: 10.1016/j.ijantimicag.2009.06.031

Oudit, G. Y., Crackower, M. A., Backx, P. H., and Penninger, J. M. (2003). The role of ACE2 in cardiovascular physiology. *Trends. Cardiovas. Med.* 13, 93–101. doi: 10.1016/S1050-1738(02)00233-5

Patel, V. B., Clarke, N., Wang, Z., Fan, D., Parajuli, N., Basu, R., et al. (2014). Angiotensin II induced proteolytic cleavage of myocardial ACE2 is mediated by TACE/ADAM-17: a positive feedback mechanism in the RAS. *J. Mol. Cell. Cardiol.* 66, 167–176. doi: 10.1016/j.yjmcc.2013.11.017

Prajapati, S. M., Patel, K. D., Vekariya, R. H., Panchal, S. N., and Patel, H. D. (2014). Recent advances in the synthesis of quinolines: a review. *Rsc. Adv.* 4, 24463–24476. doi: 10.1039/C4RA01814A

Ramanathan, S., Mathias, A. A., German, P., and Kearney, B. P. (2011). Clinical pharmacokinetic and pharmacodynamic profile of the HIV integrase inhibitor elvitegravir. *Clin. Pharmacokinet.* 50, 229–244. doi: 10.1128/AAC.25.5.633

Sams, C., Cocker, J., and Lennard, M. S. (2004). Biotransformation of chlorpyrifos and diazinon by human liver microsomes and recombinant human cytochrome P450s (CYP). *Xenobiotica* 34, 861–873. doi: 10.1080/00498250400017273

Samuelsen, O. B., Bergh, O., and Ervik, A. (2003). A single-dose pharmacokinetic study of oxolinic acid and vetoquinol, an oxolinic acid ester, in cod, *Gadus morhua* L., held in sea water at 8 degrees C and *in vitro* antibacterial activity of oxolinic acid against *Vibrio anguillarum* strains isolated from diseased cod. *J. Fish Dis.* 26, 339–347. doi: 10.1046/j.1365-2761.2003.00466.x

Sato, M., Motomura, T., Aramaki, H., Matsuda, T., Yamashita, M., Ito, Y., et al. (2006). Novel HIV-1 integrase inhibitors derived from quinolone antibiotics. *J. Med. Chem.* 49, 1506–1508. doi: 10.1021/jm0600139

Shaddinger, B. C., Xu, Y., Roger, J. H., Macphee, C. H., Handel, M., Baidoo, C. A., et al. (2014). Platelet aggregation unchanged by lipoprotein-associated phospholipase A2 inhibition: results from an *in vitro* study and two randomized phase I trials. *PloS ONE* 9:e83094. doi: 10.1371/journal.pone.0083094

Shannon, A., Le, N. T. T., Selisko, B., Eydoux, C., Alvarez, K., Guillemot, J. C., et al. (2020). Remdesivir and SARS-CoV-2: structural requirements at both nsp12 RdRp and nsp14 Exonuclease active-sites. *Antivir. Res.* 178:104793. doi: 10.1016/j.antiviral.2020.104793

Shen, M., Tian, S., Li, Y., Li, Q., Xu, X., Wang, J., et al. (2012). Drug-likeness analysis of traditional Chinese medicines: 1. Property distributions of drug-like compounds, non-drug-like compounds and natural compounds from traditional Chinese medicines. *J. Cheminformat.* 4:31. doi: 10.1186/1758-2946-4-31

Shimura, K., Kodama, E., Sakagami, Y., Matsuzaki, Y., Watanabe, W., Yamataka, K., et al. (2008). Broad antiretroviral activity and resistance profile of the novel human immunodeficiency virus integrase inhibitor elvitegravir (JTK-303/GS-9137). *J. Virol.* 82, 764–774. doi: 10.1128/JVI.01534-07

Shitara, Y. (2010). Clinical importance of OATP1B1 and OATP1B3 in drug-drug interactions. *Drug. Metab. Pharmacok.* 26, 220–227. doi: 10.2133/dmpk.DMPK-10-RV-094

Stebbing, J., Phelan, A., Griffin, I., Tucker, C., Oechsle, O., Smith, D., et al. (2020). COVID-19: combining antiviral and anti-inflammatory treatments. *Lancet. Infect. Dis.* 20, 400–402. doi: 10.1016/S1473-3099(20)30132-8

Tan, E. L., Ooi, E. E., Lin, C. Y., Tan, H. C., Ling, A. E., Lim, B., et al. (2004). Inhibition of SARS coronavirus infection *in vitro* with clinically approved antiviral drugs. *Emerg. Infect. Dis.* 10:581. doi: 10.3201/eid1004.030458

Taylor, S., Back, D. J., Drake, S. M., Workman, J., Reynolds, H., Gibbons, S. E., et al. (2001). Antiretroviral drug concentrations in semen of HIV-infected men: differential penetration of indinavir, ritonavir and saquinavir. *J. Antimicrob. Chemother.* 48, 351–354. doi: 10.1093/jac/48.3.351

Thomas-Rüddel, D., Winning, J., Dickmann, P., Ouart, D., Kortgen, A., Janssens, U., et al. (2020). Coronavirus disease 2019 "(COVID-19): update für Anästhesisten und Intensivmediziner März 2020. *Der. Anaesthesist.* 69, 225–235. doi: 10.1007/s00101-020-00758-x

Touret, F., and de Lamballerie, X. (2020). Of chloroquine and COVID-19. *Antivir. Res.* 177:104762. doi: 10.1016/j.antiviral.2020.104762

Trott, O., and Olson, A. J. (2010). AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *J. Comput. Chem.* 31, 455–461. doi: 10.1002/jcc.21334

Velkoska, E., Patel, S. K., and Burrell, L. M. (2016). Angiotensin converting enzyme 2 and diminazene: role in cardiovascular and blood pressure regulation. *Curr. Opin. Nephrol. Hypertens.* 25, 384–395. doi: 10.1097/MNH.0000000000000254

Walmsley, S., Bernstein, B., King, M., Arribas, J., Beall, G., Ruane, P., et al. (2002). Lopinavir–ritonavir versus nelfinavir for the initial treatment of HIV infection. *N. Engl. J. Med.* 346, 2039–2046. doi: 10.1056/NEJMoa012354

Wang, M., Cao, R., Zhang, L., Yang, X., Liu, J., Xu, M., et al. (2020). Remdesivir and chloroquine effectively inhibit the recently emerged novel coronavirus (2019-nCoV) *in vitro*. *Cell. Res.* 30, 269–271. doi: 10.1038/s41422-020-0282-0

Wang, Y., Zhang, D., Du, G., Du, R., Zhao, J., Jin, Y., et al. (2020). Remdesivir in adults with severe COVID-19: a randomised, double-blind, placebo-controlled, multicentre trial. *The Lancet* 395, 1569–1578. doi: 10.1016/S0140-6736(20)31022-9

Wright, H. T., Nurse, K. C., and Goldstein, D. J. (1981). Nalidixic acid, oxolinic acid, and novobiocin inhibit yeast glycyl-and leucyl-transfer RNA synthetases. *Science* 213, 455–456. doi: 10.1126/science.7017932

Wu, C., Liu, Y., Yang, Y., Zhang, P., Zhong, W., Wang, Y., et al. (2020). Analysis of therapeutic targets for SARS-CoV-2 and discovery of potential drugs by computational methods. *Acta. Pharma. Sin. B* 10, 766–788. doi: 10.1016/j.apsb.2020.02.008

Yagil, Y., Yagil, C., Crackower, M., and Penninger, J. (2003). OR-51: the protective role of ACE2 in hypertension. *Am. J. Hypertens.* 16:23A. doi: 10.1016/S0895-7061(03)00135-3

Yan, R., Zhang, Y., Li, Y., Xia, L., Guo, Y., and Zhou, Q. (2020). Structural basis for the recognition of SARS-CoV-2 by full-length human ACE2. *Science* 367, 1444–1448. doi: 10.1126/science.abb2762

Yao, X., Ye, F., Zhang, M., Cui, C., Huang, B., Niu, P., et al. (2020). *In vitro* antiviral activity and projection of optimized dosing design of hydroxychloroquine for the treatment of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). *Clin. Infect. Dis.* doi: 10.1093/cid/ciaa237

Zhang, H., Penninger, J. M., Li, Y., Zhong, N., and Slutsky, A. S. (2020). Angiotensin-converting enzyme 2 (ACE2) as a SARS-CoV-2 receptor: molecular mechanisms and potential therapeutic target. *Intensive. Care. Med.* 46, 586–590. doi: 10.1007/s00134-020-05985-9

Zhang, L., Lin, D., Sun, X., Curth, U., Drosten, C., Sauerhering, L., et al. (2020). Crystal structure of SARS-CoV-2 main protease provides a basis for design of improved α-ketoamide inhibitors. *Science* 368, 409–412. doi: 10.1126/science.abb3405

Zhao, Y., Zhao, Z., Wang, Y., Zhou, Y., Ma, Y., and Zuo, W. (2020). Single-cell RNA expression profiling of ACE2, the receptor of SARS-CoV-2. *Biorxiv [Preprint]*. doi: 10.1101/2020.01.26.919985

Zou, L., Ruan, F., Huang, M., Liang, L., Huang, H., Hong, Z., et al. (2020). SARS-CoV-2 viral load in upper respiratory specimens of infected patients. *N. Engl. J. Med.* 382, 1177–1179. doi: 10.1056/NEJMc2001737

Zumla, A., Chan, J. F., Azhar, E. I., Hui, D. S., and Yuen, K. Y. (2016). Coronaviruses—drug discovery and therapeutic options. *Nat. Rev. Drug Discov.* 15:327. doi: 10.1038/nrd.2015.37

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Big Data

BRIEF RESEARCH REPORT
published: 24 July 2020
doi: 10.3389/fdata.2020.00026

Novel Criteria for When and How to Exit a COVID-19 Pandemic Lockdown

Chenyu Li[1], *Paola Romagnani*[2] *and Hans-Joachim Anders*[1]*

[1] Medizinische Klinik und Poliklinik IV, Klinikum der Universität, Ludwig-Maximilians-Universität München, Munich, Germany, [2] Excellence Centre for Research, Transfer and High Education for the Development of DE NOVO Therapies (M.E.M., P.R.), University of Florence, Florence, Italy

In the first month of 2020, severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), a novel coronavirus spreading quickly via human-to-human transmission, caused the coronavirus disease 2019 (COVID-19) pandemic. Italy installed a successful nationwide lockdown to mitigate the exponential increase of case numbers, as the basic reproduction number R0 reached 1 within 4 weeks. But is R0 really the relevant criterion as to whether or not community spreading is under control? In most parts of the world, testing largely focused on symptomatic cases, and we thus hypothesized that the true number of infected cases and relative testing capacity are better determinants to guide lockdown exit strategies. We employed the SEIR model to estimate the numbers of undocumented cases. As expected, the estimated numbers of all cases largely exceeded the reported ones in all Italian regions. Next, we used the numbers of reported and estimated cases per million of population and compared it with the respective numbers of tests. In Lombardy, as the most affected region, testing capacity per reported new case seemed between two and eight most of the time, but testing capacity per estimated new cases never reached four up to April 30. In contrast, Veneto's testing capacity per reported and estimated new cases were much less discrepant and were between four and 16 most of the time. As per April 30 also Marche, Lazio and other Italian regions arrived close to 16 ratio of test capacity per new estimated infection. Thus, the criterion to exit a lockdown should be decided at the level of the regions, based on the local testing capacity that should reach 16 times the estimated true number of newly infected cases as predicted.

Keywords: COVID-19, SARS-CoV-2, SEIR epidemic model, basic reproduction number, lockdown measures

OPEN ACCESS

Edited by:
Weida Tong,
National Center for Toxicological Research (FDA), United States

Reviewed by:
Dong Wang,
National Center for Toxicological Research (FDA), United States
Mary Yang,
University of Arkansas at Little Rock, United States

***Correspondence:**
Hans-Joachim Anders
hjanders@med.uni-muenchen.de

Specialty section:
This article was submitted to Medicine and Public Health, a section of the journal Frontiers in Big Data

Received: *07 May 2020*
Accepted: *09 July 2020*
Published: *24 July 2020*

Citation:
Li C, Romagnani P and Anders H-J (2020) Novel Criteria for When and How to Exit a COVID-19 Pandemic Lockdown. Front. Big Data 3:26. doi: 10.3389/fdata.2020.00026

INTRODUCTION

In the first month of 2020, severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), a novel coronavirus spreading quickly via human-to-human transmission, caused the coronavirus disease 2019 (COVID-19) pandemic. In most countries, the disease started from few cases in one province or area and, depending on the efficacy of immediate containment measures, remained under control or lead to uncontrolled community transmission. In case early containment measures were not sufficient, the local outbreak turned into uncontrolled community transmission (Leung et al., 2020), ultimately addressed by social distancing and, in some cases, complete lockdown (Li C. et al., 2020). However, such mitigation measures come at large costs in terms of declining economic activity, employment rates, and wealth of a nation. Increasing depts, poverty, domestic violence, and mental health problems are

only some of the economic and social consequences of such mitigation measures. In expectation of these trade-offs, when and how to install mitigation measures is a matter of debate among decision-makers. The same debate later occurred with regards to when and how one can implement the installed mitigation. Some countries installed different measures in each region depending on the extent to which COVID-19 affected the respective region. Not so for Italy.

In February 2020, Italy was the first country in Europe noting local outbreaks; these were in Veneto and Lombardy, two regions in the northeast and northwest of Italy, respectively, and, while early containment measures controlled the problem in Veneto, the infection spread in an uncontrolled manner in Lombardy. On March 8, the Italian government installed a nationwide lockdown during a moment where symptomatic COVID-19 was highly prevalent in Lombardy, while many other regions of Italy had seen few cases. This offers the unique possibility of analyzing the effect of identical mitigation measures on different phases of community spreading of COVID-19 using real world data.

MATERIALS AND METHODS

Data Source

The data of tested, confirmed, hospitalized, and deceased cases of SARS-CoV-2 reported by provinces in Italy were obtained from the Italian Ministry of Health (Ministero della Salute, http://www.salute.gov.it/portale/nuovocoronavirus/homeNuovoCoronavirus.jsp?).

Susceptible Exposed Infectious Recovered Model

We proposed a deterministic "Susceptible-Exposed-Infectious-Recovered" (SEIR) compartmental model based on the clinical disease severity and intervention measures. For the modified SEIR model, the population under consideration was stratified by six groups as susceptible (S), exposed (E), mild infectious (I), hospitalized (H), recovered (R), and deceased (D) compartments.

$$\begin{aligned}
\frac{dS(t)}{dt} &= -\frac{\beta S(t) I(t)}{N} \\
\frac{dE(t)}{dt} &= \frac{\beta S(t) I(t)}{N} - \alpha E(t) \\
\frac{dI(t)}{dt} &= \alpha E(t) - (\gamma + p) I \\
\frac{dH(t)}{dt} &= pI(t) - (\gamma_h + \mu) H(t) \\
\frac{dR(t)}{dt} &= \gamma I(t) + \gamma_h H(t) \\
\frac{dD(t)}{dt} &= \mu H(t)
\end{aligned}$$

The model was parameterized by using data obtained for the previous report of SARS-CoV-2, where β is the force of infection or disease transmission rate, α is the inverse of the latent period (days), $(\gamma + p)$ is the inverse of the mild infectious period (days) or removal rate, p is the rate of mild cases progress to severe cases requiring hospitalization, $(\gamma_h + \mu)$ is the removal rate from hospitalization, and μ is the mortality rate for SARS-CoV-2 inpatient. Parameters are summarized in **Table 1**.

TABLE 1 | Parameters of the susceptible-exposed-infected-removed model.

Quantity	Parameter	Value	Source
Basic reproduction number	R0	2.2 (1.6–3.0)	Kucharski et al., 2020; Li Q. et al., 2020; Wu et al., 2020; Zhao et al., 2020
Average incubation period	$\frac{1}{\alpha}$	5 days	Lauer et al., 2020
Average duration of mild infection	$\frac{1}{(\gamma + p)}$	6 days	Prem et al., 2020
Proportion of severe infections	$\frac{p}{(\gamma + p)}$	15%	Wu and McGoogan, 2020
Average time from onset of symptoms to death	–	18 days	Verity et al., 2020
Average Duration of hospitalization	$\frac{1}{(\gamma_h + \mu)}$	12 days	[Average time from onset of symptoms to death]- [Average duration of mild infection]
Case fatality ratio	$\frac{Server\% \times \mu}{(\gamma_h + \mu)}$	2.2–3.3%	Bassetti et al., 2020; Russell et al., 2020; Verity et al., 2020; Wang et al., 2020

Estimation of Infected Cases and Basic Reproductive Number

Instead of the number of SARS-CoV-2-positive individuals reported by authorities, often falsely referred to as "infected cases" because they mostly represent the capability and intensity of testing activity, we employed the numbers of deceased cases. They provide a more robust estimate of outbreak trends, especially when the number of infected individuals exceeds by far the number of those tested positive. To reversely estimate the number of infected cases based on deceased cases number, we used cubic spline with a smoothing parameter of 0.6 to reduce the data noise of deceased cases and then calculated the number of hospitalized cases at time t, $H(t) = \frac{D_{t+1} - D_t}{\mu}$, infected cases with mild symptom number at time t, $I(t) = \frac{H_{t+1} + (\gamma_h + \mu - 1) H_t}{p}$, and new recovered cases number at time t, $R_{new}(t) = \gamma I_t + \gamma_h H_t$. All together, the total number of infected cases estimates at time t is: $I^{total}(t) = I(t) + H(t) + D(t) + \sum_{i=1}^{t} R_{new}(i)$. The reported hospitalized cases number was also used to estimate infected cases number by same strategy.

We assumed that, during the early phase, before depletion of susceptible individuals, the curve of infected individuals should follow an exponential increase with basic reproductive number (R0) = 2.5 as previously reported (Hellewell et al., 2020; Zhao et al., 2020). Upon installment of mitigation measures, a real-time reproductive number (*Rt)* was calculated according to a Bayesian framework algorithm established by Thompson et al. (Thompson et al., 2019). The probability of occurrence of a case

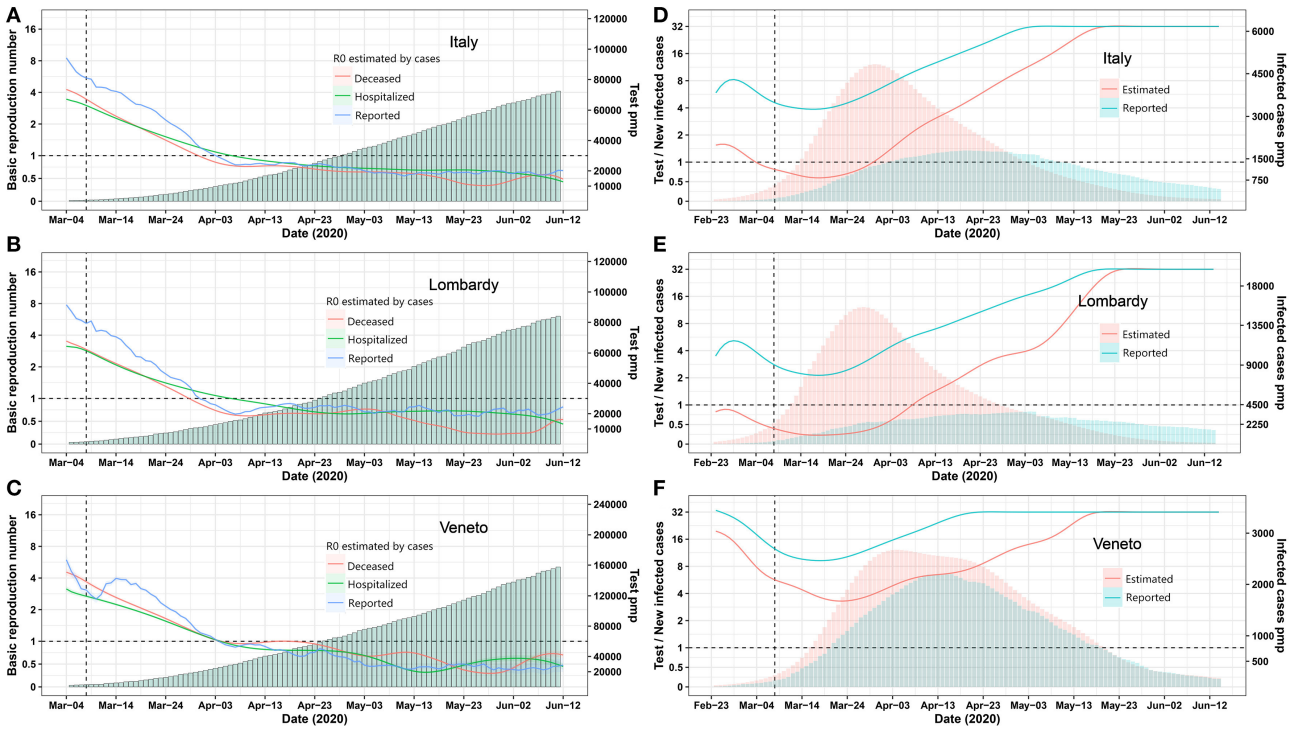


FIGURE 1 | Epidemic trends for Italy and its provinces Lombardia and Veneto. **(A–C)** Real-time R0 and test per million. The blue, green, and red curve represents R0 estimated by using Bayesian framework algorithm based on reported, hospitalized, and deceased cases. The green bars represent test per million. **(D–F)** Daily test/estimated and reported infected cases. The green and red bars represent reported and estimated infected number. The green and red curves represent the number of daily test/reported and estimated infected cases. A vertical dashed line indicates the nationwide lockdown on March 8. Pmp, per million of population. All data were obtained from the Italian Ministry of Health (Ministero della Salute, http://www.salute.gov.it/portale/nuovocoronavirus/homeNuovoCoronavirus.jsp?).

was expressed as

$$P\left(I_{t-\tau}, I_{t-\tau+1}, \ldots, I_t | I_0, I_1, \ldots, I_{t-1}, W_s, R_t\right) = \prod_{K=t-\tau}^{t} \frac{\left(R_t \Lambda_k\left(W_s\right)\right)^{I_k} exp\left(-R_t \Lambda_k\left(W_s\right)\right)}{I_k!}$$

where Λ_k represents the number of total infected individuals at time k, τ (7 days) represents the length of the time window over which Rt is estimated, and W_s is the serial interval distribution. Then we used a gamma distribution prior and conjugating to the Poisson likelihood to obtain an analytical formulation of the posterior distribution of Rt (Thompson et al., 2019). In addition to estimate Rt based on the reported infected cases, we also performed calculations using decease-estimated infected cases.

All analyses were performed using R software (version 3.6.1). *EpiEstim* package was used to implement Rt algorithm (Thompson et al., 2019).

RESULTS

Italy Lockdown

On March 8, 2020, Italy installed a nationwide lockdown to mitigate the exponential increase of case numbers. We assessed its effect ex post by calculating the real-time R0 based on the reported tested positive cases and deceased cases to understand the dynamic changes of infection spreading. Above all, the Italian lockdown measures were successful, as the real-time basic reproduction number R0 for infected, hospitalized, and deceased cases were decreasing in a parallel manner and reached 1 on March 22, which meant the epidemic come under control. In most regions of Italy, the R0 declined to <1 within 4 weeks of lockdown (**Figures 1A–C**, **Figure S1**), but is R0 really the relevant criterion with which to determine whether or not community spreading is under control?

Novel Criteria

Italy ramped up testing capacities to isolate infected individuals but again to a much different extent as per million of population in each region (**Figures 1A–C**, **Figure S1**). In most parts of the world, and thus most regions of Italy, testing largely focused on symptomatic cases, ignoring that the pandemic spreads via unrecognized asymptomatic individuals (Li R. et al., 2020). Therefore, we hypothesized that the true number of infected cases and relative testing capacity are better determinants to guide lockdown exit strategies and, because these parameters likely differ in each region, may suggest different exit strategies in each region.

We employed the "Susceptible-Exposed-Infectious-Recovered" (SEIR) model to estimate the numbers of all infected cases for each Italian region on the basis of reported

deceased cases as these are more reliable (**Figure S2**). The prediction model was reliable, as predicted and reported numbers of hospitalized and deceased COVID-19 cases matched very well for most regions (**Figure S2**). As expected, the estimated numbers of all infected cases largely exceeded the reported ones in all regions (**Tables S1, S2**). Next, we used the numbers of reported and estimated cases per million of population and compared it with the respective numbers of tests (**Figure 1D**, **Figure S3**). In Lombardy, as the most affected region, testing capacity per reported new case seemed between two to eight most of the time, but testing capacity per estimated new cases never reached four up to April 30 (**Figure 1E**). In contrast, Veneto's testing capacity per reported and estimated new cases were much less discrepant and were between four and 16 most of the time (**Figure 1F**). As per April 30, Marche, Lazio, Campania, Puglia, Friuli Venezia, Giulia Sicilia, Umbria, Calabria, Basilicata, Liguria, and Veneto also arrived close to 16 ratio of test capacity per new estimated infection (**Figure S3**). Thus, the criterion to exit a lockdown should be decided at the level of the regions, based on the local testing capacity that should reach 16 times the estimated true number of newly infected cases as predicted.

DISCUSSION

The timing of reopening could be a complex and step-by-step issue, which needs to balance the local capacity to identify infected cases and the degree of social contact. Therefore, the question is how many people contact infected cases per day, and how many could get a test. The concept of testing/new cases is more like a parameter to assess the capacity for authorities to trace the potential cases exposed by one infected case. For example, the testing capacity is 16 times the new cases, which means 16 exposed cases get tested per newly infected case, and the number 16 is about equal to the number of people contacted per infected cases in lockdown setting. However, the number should be increased if we reopen since people have more chance to contact with others

On May 18, Italy reopened commercial activities—all regions' testing/new cases reached 16 ratios. Since this partial reopening, the epidemic remains under control without any subsequent adverse consequence, which supports our conclusion. With the continuous increase in testing capabilities, the number of infected cases is declining, and a full reopening is just around the corner.

A nationwide exit from lockdown would ignore that the capacity to control community spreading differs across regions, which is not sufficiently indicated by the basic reproduction number R0 (Hellewell et al., 2020). Thus, when and how to exit a lockdown should be decided at the level of the regions, or potentially even on a district level, based on the local testing capacity that should reach 16 times the estimated true number of newly infected cases as predicted, e.g., by the deceased cases in this district or region. Reaching congruency between estimated and documented cases and a sufficient capacity to isolate new cases are further requirements. Based on these indications, regions like, for example, Veneto, Campania, Friuli Venezia Giulia, Umbria, Calabria, Basilicata, or Sardegna may exit some of the lockdown measures earlier than Lombardy, Emilia-Romagna, or Piemonte if travel restrictions across the regions remain in place.

We believe there are not enough data to draw relevant conclusion about the consequence of a region being reopened before certain criteria are met, while, in our opinion, a test capacity of 16 ratios per new estimated infection is a robust criterion for the authorities to consider further strategies of exiting lockdown gradually. This model can help in making political decision also in other countries or regions of the world, provided that the necessary data are available at the regional or district level.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

CL, PR, and H-JA conceived and designed the study. CL performed the statistical analysis and wrote the paper. PR and H-JA reviewed and edited the manuscript. All authors read and approved the manuscript.

FUNDING

H-JA was supported by the Deutsche Forschungsgemeinschaft (AN372/24-1). No funding bodies had any role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fdata.2020.00026/full#supplementary-material

REFERENCES

Bassetti, M., Vena, A., and Giacobbe, D. R. (2020). The novel Chinese coronavirus (2019-nCoV) infections: challenges for fighting the storm. *Eur. J. Clin. Invest.* 50:e13209. doi: 10.1111/eci.13209

Hellewell, J., Abbott, S., Gimma, A., Bosse, N. I., Jarvis, C. I., Russell, T. W., et al. (2020). Feasibility of controlling COVID-19 outbreaks by isolation of cases and contacts. *Lancet Glob. Health* 8, e488–e496. doi: 10.1016/S2214-109X(20)30074-7

Kucharski, A. J., Russell, T. W., Diamond, C., Liu, Y., Edmunds, J., Funk, S., et al. (2020). Early dynamics of transmission and control of COVID-19: a mathematical modelling study. *Lancet Infect. Dis.* 20, 553–558. doi: 10.1016/S1473-3099(20)30144-4

Lauer, S. A., Grantz, K. H., Bi, Q., Jones, F. K., Zheng, Q., Meredith, H. R., et al. (2020). The incubation period of coronavirus disease 2019 (COVID-19) from publicly reported confirmed cases: estimation and application. *Ann. Intern. Med*. 172, 577–582. doi: 10.7326/M20-0504

Leung, K., Wu, J. T., Liu, D., and Leung, G. M. (2020). First-wave COVID-19 transmissibility and severity in China outside Hubei after control measures, and second-wave scenario planning: a modelling impact assessment. *Lancet* 395, 1382–1393. doi: 10.1016/S0140-6736(20)30746-7

Li, C., Romagnani, P., von Brunn, A., and Anders, H. J. (2020). SARS-CoV-2 and Europe: timing of containment measures for outbreak control. *Infection* 48, 483–486. doi: 10.1007/s15010-020-01420-9

Li, Q., Guan, X., Wu, P., Wang, X., Zhou, L., Tong, Y., et al. (2020). Early transmission dynamics in Wuhan, China, of novel coronavirus-infected pneumonia. *N. Engl. J. Med.* 382, 1199–1207. doi: 10.1056/NEJMoa2001316

Li, R., Pei, S., Chen, B., Song, Y., Zhang, T., Yang, W., et al. (2020). Substantial undocumented infection facilitates the rapid dissemination of novel coronavirus (SARS-CoV2). *Science* 368, 489–493. doi: 10.1126/science.abb3221

Prem, K., Liu, Y., Russell, T. W., Kucharski, A. J., Eggo, R. M., Davies, N., et al. (2020). The effect of control strategies to reduce social mixing on outcomes of the COVID-19 epidemic in Wuhan, China: a modelling study. *Lancet Public Health* 5, e261–e270. doi: 10.1016/S2468-2667(20)30073-6

Russell, T. W., Hellewell, J., Jarvis, C. I., van Zandvoort, K., Abbott, S., Ratnayake, R., et al. (2020). Estimating the infection and case fatality ratio for coronavirus disease (COVID-19) using age-adjusted data from the outbreak on the Diamond Princess cruise ship, February 2020. *Euro Surveill.* 25:2000256. doi: 10.2807/1560-7917.ES.2020.25.12.2000256

Thompson, R. N., Stockwin, J. E., van Gaalen, R. D., Polonsky, J. A., Kamvar, Z. N., Demarsh, P. A., et al. (2019). Improved inference of time-varying reproduction numbers during infectious disease outbreaks. *Epidemics* 29:100356. doi: 10.1016/j.epidem.2019.100356

Verity, R., Okell, L. C., Dorigatti, I., Winskill, P., Whittaker, C., Imai, N., et al. (2020). Estimates of the severity of coronavirus disease 2019: a model-based analysis. *Lancet Infect. Dis*. 20, 669–677. doi: 10.1016/S1473-3099(20)30243-7

Wang, W., Tang, J., and Wei, F. (2020). Updated understanding of the outbreak of 2019 novel coronavirus (2019-nCoV) in Wuhan, China. *J. Med. Virol*. 92, 441–447. doi: 10.1002/jmv.25689

Wu, J. T., Leung, K., and Leung, G. M. (2020). Nowcasting and forecasting the potential domestic and international spread of the 2019-nCoV outbreak originating in Wuhan, China: a modelling study. *Lancet* 395, 689–697. doi: 10.1016/S0140-6736(20)30260-9

Wu, Z., and McGoogan, J. M. (2020). Characteristics of and important lessons from the coronavirus disease 2019 (COVID-19) outbreak in China: summary of a report of 72314 cases from the Chinese Center for Disease Control and Prevention. *JAMA* 323, 1239–1242. doi: 10.1001/jama.2020.2648

Zhao, S., Musa, S. S., Lin, Q., Ran, J., Yang, G., Wang, W., et al. (2020). Estimating the unreported number of novel coronavirus (2019-nCoV) cases in china in the first half of January 2020: a data-driven modelling analysis of the early outbreak. *J. Clin. Med.* 9:388. doi: 10.3390/jcm9020388

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Public Health

ORIGINAL RESEARCH
published: 28 July 2020
doi: 10.3389/fpubh.2020.00419

Assessment of Healthcare System Capabilities and Preparedness in Yemen to Confront the Novel Coronavirus 2019 (COVID-19) Outbreak: A Perspective of Healthcare Workers

Mohammed Zawiah[1,2], Fahmi Y. Al-Ashwal[1], Ramzi Mukred Saeed[3,4], Mohammed Kubas[5,6], Sara Saeed[7], Amer Hayat Khan[1], Syed Azhar Syed Sulaiman[1,8] and Rami Abduljabbar[3,4]*

[1] Department of Clinical Pharmacy, School of Pharmaceutical Sciences, Universiti Sains Malaysia, Gelugor, Malaysia, [2] Department of Pharmacy Practice, College of Clinical Pharmacy, University of Al Hodeida, Al Hodeida, Yemen, [3] Department of Biopharmaceutics and Clinical Pharmacy, School of Pharmacy, The University of Jordan, Amman, Jordan, [4] Department of Clinical Pharmacy and Pharmacy Practice, University of Science and Technology (UST), Sana'a, Yemen, [5] Clinical Pharmacy Department, University of Science and Technology Hospital (USTH), Sana'a, Yemen, [6] Pharmacy Practice Department, Kulliyyah of Pharmacy, International Islamic University Malaysia (IIUM), Kuantan, Malaysia, [7] School of Dentistry, The University of Jordan, Amman, Jordan, [8] Advanced Medical and Dental Institute, Universiti Sains Malaysia, Kepala Batas, Malaysia

OPEN ACCESS

Edited by:
Alexander Rodriguez-Palacios, Case Western Reserve University, United States

Reviewed by:
Hatem Elshabrawy, Sam Houston State University, United States
Helena Maltezou, Hellenic Center for Disease Control & Prevention, Greece

***Correspondence:**
Ramzi Mukred Saeed
ramzimokred@gamil.com

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Public Health

Received: *06 May 2020*
Accepted: *13 July 2020*
Published: *28 July 2020*

Citation:
Zawiah M, Al-Ashwal FY, Saeed RM, Kubas M, Saeed S, Khan AH, Sulaiman SAS and Abduljabbar R (2020) Assessment of Healthcare System Capabilities and Preparedness in Yemen to Confront the Novel Coronavirus 2019 (COVID-19) Outbreak: A Perspective of Healthcare Workers. Front. Public Health 8:419. doi: 10.3389/fpubh.2020.00419

Background: In the past decade, Yemen has witnessed several disasters that resulted in a crumbled healthcare system. With the declaration of COVID-19 a global pandemic, and later the appearance of first confirmed cases in Yemen, there is an urgent need to assess the preparedness of healthcare facilities (HCFs) and their capacities to tackle a looming COVID-19 outbreak. Herein, we present an assessment of the current state of preparedness and capabilities of HCFs in Yemen to prevent and manage the COVID-19 outbreak.

Methods: An online survey for HCFs was developed, validated, and distributed. The questionnaire is divided into five main sections: (1) Demographic variables for participants. (2) HCFs capabilities for COVID-19 outbreak. (3) Support received to face the emergence and spread of COVID-19. (4). Current practices of infection prevention and control measures in the HCFs. The last section focused on the recommendations to ensure effective and timely response to this outbreak in Yemen. Descriptive analysis was used to analyze data using statistical package for social sciences (SPSS), version 23.

Results: Responses were received from healthcare workers (HCWs) from 18 out of 22 governorates in Yemen. Out of the 296 HCWs who participated in the study, the vast majority (93.9%) believed that the healthcare system in Yemen does not have the resources and capabilities to face and manage a COVID-19 outbreak. Approximately 82.4% of participants rated the general preparedness level of their HCFs as very poor or poor. More specifically, the majority of HCWs rated their HCFs as very poor or poor in term of availability of the following: an adequate number of mechanical ventilators (88.8%), diagnostic devices (88.2%), ICU rooms and beds (81.4%), and isolation rooms (79.7%).

Conclusions: The healthcare facilities in Yemen are unprepared and lack the most basic resources and capabilities to cope with or tackle a COVID-19 outbreak. With the current state of a fragile healthcare system, a widespread outbreak of COVID-19 in Yemen could result in devastating consequences. There is an urgent need to provide support to the healthcare workers and HCFs that are on the frontline against COVID-19.

Keywords: Yemen, COVID-19, healthcare facilities, capabilities, preparedness

INTRODUCTION

The novel coronavirus (COVID-19) has been declared by the World Health Organization (WHO) as a public health emergency of international concern on January 30, 2020 (1). A few weeks later, on March 11, 2020, WHO declared the COVID-19 outbreak a global pandemic, after the novel coronavirus infected 118,000 individuals in 114 countries (2). As of April 30, 2020, nearly every country in the world has been affected by the virus, and the WHO situation analysis of COVID-19 reported 3,090,445 confirmed cases with 217,769 deaths globally (3). In war-torn Yemen, the first confirmed case of COVID-19 was announced on April 10, 2020, in Hadramout, Yemen's largest province (4). Three weeks later, six confirmed cases of COVID-19 were reported in Yemen, with two deaths (3). The risk of larger outbreaks in Yemen is very high, given the ongoing war and conflicts, political instability and fragmentation, and its fragile health system, where only 45% of the healthcare facilities are fully functioning. The situation in Yemen is further complicated by the presence of high numbers of migrants, refugees and internal displacement of people (IDPs), and concomitant outbreaks of communicable diseases such as cholera, dengue, and diphtheria (4–8). Yemen remains the world's largest humanitarian crisis, with nearly 80 percent of the population requiring some form of humanitarian assistance and protection (4).

The healthcare system in Yemen is largely dependent on the support of international organizations (9). There are 39 health cluster partners [UN agencies, international non-government organizations (NGOs), and national NGOs] that provide support to the primary and secondary healthcare services across the country as of December 2019 (10). However, many gaps in the healthcare system still exist, and the capability and capacity of Yemen's healthcare facilities for facing a widespread COVID-19 outbreak is unknown. Therefore, healthcare facilities (HCFs) preparedness for emergency response and capacities for COVID-19 outbreaks needs to be ascertained. In this study, we describe the current state of emergency response and preparedness for facing COVID-19 in Yemen's healthcare facilities. The study provides a baseline level for preparedness and capacities of the HCFs for facing COVID-19 and allows for future comparative work and intervention progress assessment. Moreover, the results could be utilized by healthcare policy-makers and health cluster partners in designing and providing the appropriate interventions to urgently enhance the preparedness and competency of the HCFs in Yemen, and to ensure their readiness to launch an effective response to prevent, control and manage COVID-19 and future outbreaks.

METHODS

Study Design and Setting

A cross-sectional study using an online survey-based questionnaire was conducted in Yemen over a period of 2 weeks, opened on March 27, 2020, and closed on April 9, 2020, a day before Yemen's first COVID-19 was revealed. Eligible participants were healthcare workers (HCWs) and administrative personnel working at governmental, private, and non-governmental organizations (NGOs) hospitals. Eighteen out of twenty-two governorates were covered in this survey. Ethical approval was approved by the institutional review board committee at the University of Sciences and Technology, Sana'a, Yemen (ECA/UST189).

Instrument

An online survey was developed, validated, and distributed to the targeted population. The original draft of the questionnaire was evaluated for face validity by four independent healthcare providers, and modifications were made where appropriate according to the comments and feedback provided. The final version of the questionnaire included five main sections addressing various topics of interest. The first section of this study was demographic data intended to elicit information to describe the respondent. The second part contained: (1) a close-ended question (Yes/No) about whether the healthcare system in Yemen is prepared or not for COVID-19 outbreak; (2) a general Likert-type question (very poor, poor, fair, good, and very good) to rate the capability and preparedness of the healthcare facility they are working in to face COVID-19; (3) 10 specific questions addressing the preparedness level of their healthcare facilities (HCFs). In these 10 questions, HCWs were asked about how their HCFs are prepared in terms of 10 essential competencies for managing the COVID-19 outbreak, including diagnostic devices, mechanical ventilators, intensive care unit rooms and beds, private isolation rooms, personal protective equipment, sufficient trained personnel, adequate knowledge, enough beds in all departments, alternative electricity source, and pre-emptive plans. The third section was about the support received to face the outbreak. Section four assessed the current practices of infection prevention and control measures in the HCFs. The last section addressed the recommendations that should be made to respond to this outbreak in Yemen.

Survey Implementation and Analysis

Participants were recruited using social media such as Facebook Messengers and WhatsApp; those willing to participate could

TABLE 1 | Healthcare providers' characteristics.

Category	Subcategory	F (%)/Mean (SD)
Gender	Male	240 (81.4)
	Female	56 (18.6)
Age, Years	Mean ± SD	32.9 ± 7.4
	Minimum	20
	Maximum	72
Experience, Years	Mean ± SD	7.9 ± 6.5
	Minimum	1
	Maximum	46
Specialty	Consultant	15 (5.1)
	Specialist	67 (22.6)
	GP	74 (25.0)
	Nurse	26 (8.8)
	Hospital pharmacist	60 (20.3)
	Laboratory technician	16 (5.4)
	Physician assistant	14 (4.7)
	Administration	24 (8.1)
Department	Emergency	52 (17.6)
	ICU	29 (8.9)
	Pediatric	18 (6.1)
	General/Family medicine	15 (5.1)
	Infectious diseases	10 (3.4)
	Respiratory	7 (2.4)
	Others	165 (55.7)
Working place	Governmental hospital	133 (44.9)
	Private hospital	127 (42.9)
	NGO hospital	36 (12.2)
Governorate	Sana'a	130 (43.9)
	Aden	38 (12.8)
	Taiz	26 (8.8)
	Ib	21 (7.1)
	Al Hodeida	20 (6.8)
	Others	61 (20.6)

SD, standard deviation; ICU, intensive care unit; NGOs, non-governmental organization.

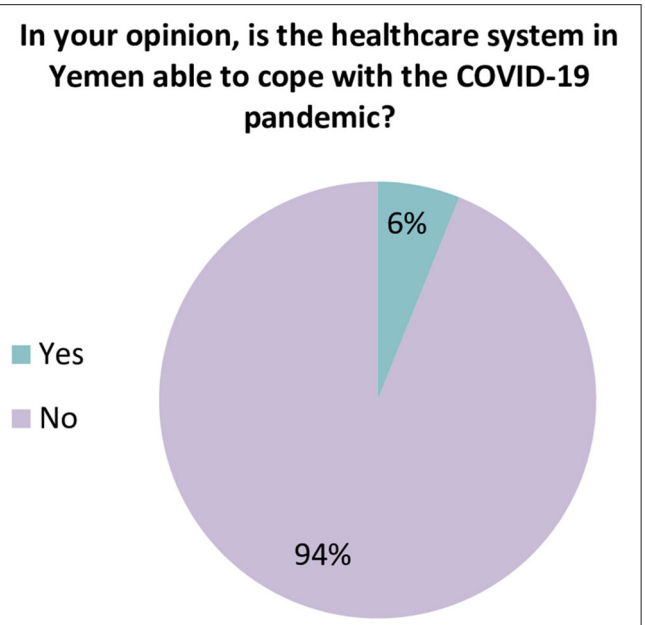


FIGURE 1 | Healthcare workers' perspective regarding the general preparedness of the healthcare system in Yemen.

open a link to initially view the consent form of the study and then proceeding to the survey. Data were collected and aggregated into Microsoft Excel file, exported into statistical package for social science (SPSS) version 21 (SPSS Inc., Chicago, IL, USA), and then analyzed. Descriptive statistics were undertaken using frequency and percentage for qualitative variables and mean and standard deviations for continuous variables. The distribution of various variables was summarized in tables and figures. Chi-square test was used to investigate the differences in preparedness and practice between demographic factors such as hospital types and departments. $P < 0.05$ was considered as statistically significant.

RESULTS

Responses were received from healthcare workers (HCWs) living in 18 out of 22 governorates of Yemen. The average age of the HCWs (296) that participated in the study was 32.9 years [standard deviation (*SD*): 7.4, range: 20–72], and 240 (81.4%) were male. The majority of participants were general practitioners (25%), specialists (22.6%), and hospital pharmacists (20.3%). Other respondents included nurses (8.8%), individuals with administrative duties (8.1%), laboratory technicians (5.4%), consultants (5.1%), and physician assistants (4.7%). Self-reported years of experience ranged from 1 to 46 years, with the average (SD) being 7.9 (6.5) years. One hundred thirty-three (44.9%) are working in governmental hospitals, 127 (42.9%) in private hospitals, and 36 (12.2%) in NGOs hospitals. Other characteristics are shown in **Table 1**.

When participants were asked about the readiness of the healthcare system in Yemen, the vast majority of HCWs (93.9%) believed that the current healthcare system in Yemen does not have the resources or capabilities to face and manage the COVID-19 outbreak (**Figure 1**). Approximately 82.4% of participants rated the general preparedness level of their HCFs as very poor or poor (**Figure 2**). More specifically, the majority of HCWs rated their HCFs as very poor or poor in term of availability of the following: an adequate number of mechanical ventilators (88.8%), diagnostic devices (88.2%), ICU rooms and beds (81.4%), and isolation rooms (79.7%) (**Figure 3**). There was a significant difference between hospitals' types in only one preparedness parameter. Governmental hospitals had a much lower level of preparedness in terms of safety equipment in comparison to NGOs and private hospitals, with poor preparedness percentages of 72.9, 52.8, and 55.9%, respectively ($p = 0.018$).

Regarding the support received from local authorities and NGOs, most HCWs (68.6%) indicated that they did not receive proper training in all aspects related to COVID-19. In this light, a large proportion of participants (66.6%) reported that they had not been trained on isolation procedures. Moreover, half of HCWs indicated that their

HCFs did not receive adequate financial support earmarked for addressing and facing COVID-19 pandemic, neither from the local health authorities nor NGOs or international agencies (**Figure 4**).

With respect to the preventive measures taken by the HCFs to limit and slow the spread of COVID-19, a large proportion of respondents (80.1%) indicated that their HCFs did not implement a social distance strategy, did not measure the temperature of patients and visitor at the entry points of their HCFs (73.3%), and did not have volunteers or employees at the entrance of the hospital to inform and educate the visitors and patients about COVID-19 best practices and preventive measures (72.3%) (**Figure 5**). There were no significant differences in the practice of preventive measures across hospital departments. However, significant differences were noted between hospitals' types. In this light, the practice of body temperature measurement and the availability of hand sanitizers at all entry points of hospitals were significantly lower in governmental hospitals compared to private and NGOs hospitals with p-values of 0.04 and <0.0001, respectively. Similarly, the availability of masks and hand sanitizers in the examination area was much lower in governmental hospitals than other hospitals ($p < 0.0001$).

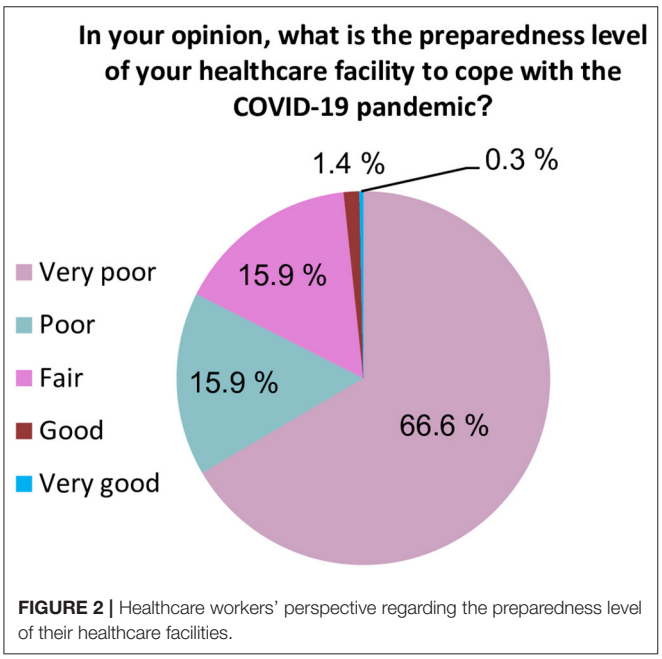


FIGURE 2 | Healthcare workers' perspective regarding the preparedness level of their healthcare facilities.

With regards to recommendations, majority of respondents recommended that (1) HCPs should be trained in all aspects of emergency response for COVID-19 outbreak (86.5%); (2) more support is urgently needed for HCFs in the form of diagnostic devices, mechanical ventilators, and adequate protective equipment (84.1%), and (3) financial aid for the HCWs and HCFs to face the outbreak (82.4%) (**Figure 6**). Some specific recommendations are highlighted below:

- Forming an independent emergency committee of individuals who are not affiliated with any political party to manage the COVID-19 crisis.
- Financial support should be directed to the health care facilities under the supervision of independent organizations.
- Payment of salaries on time and giving financial incentives for all healthcare workers.
- Daily wages workers affected by the infection-control policy should be supported financially.
- Constructing field hospitals to face COVID-19 outbreak.
- Making a management protocol for COVID-19 based on the latest evidence.
- Awareness campaigns for the community using different tools.

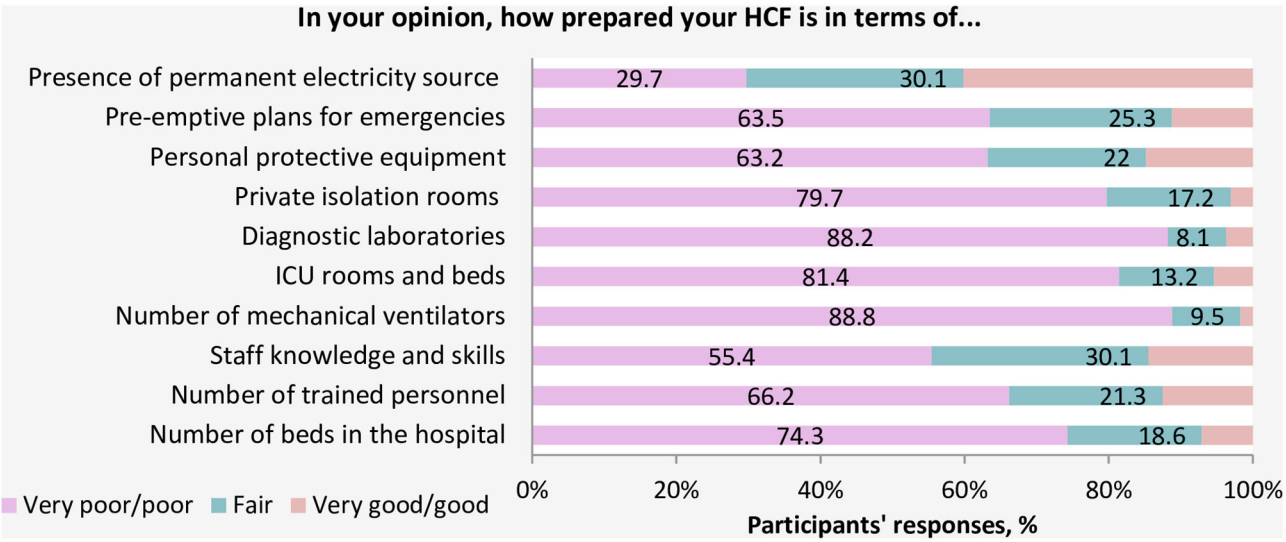


FIGURE 3 | Assessment of essential competencies for HCFs to face COVID-19.

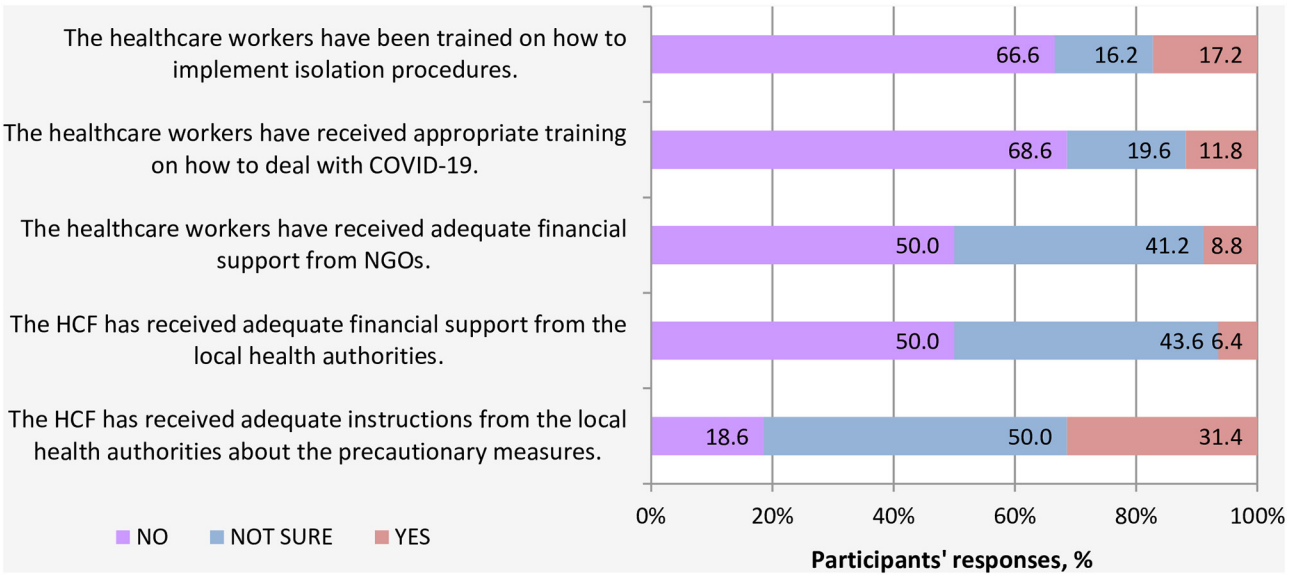


FIGURE 4 | Support received by HCFs to cope with the COVID-19 outbreak.

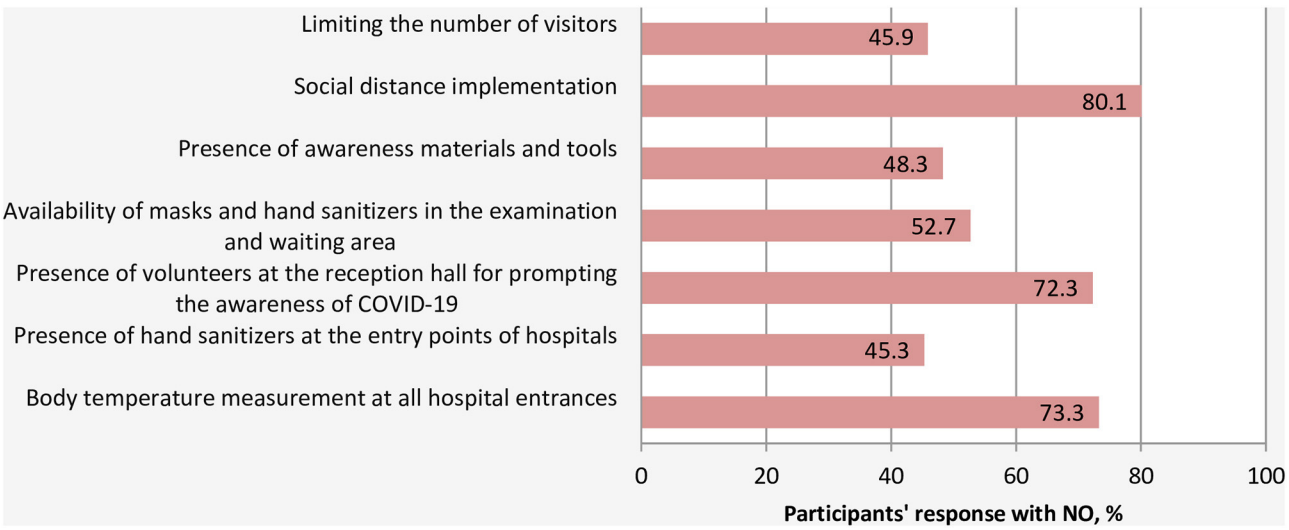


FIGURE 5 | Current practices of preventive and control measures by the HCFs.

DISCUSSION

Although various measures, by international and national authorities, are ongoing in Yemen to suppress the spread of the outbreak (11), nevertheless, the healthcare system capability and preparedness to combat COVID-19 is still unknown. Several cases of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection have been reported in the Arabian Peninsula and Middle Eastern region (Saudi Arabia, Egypt, Oman, and Jordan) during the time of data collection of this work with no reported case in Yemen (12). Yemen shares borders with some of these countries, with many people entering the country daily from these borders. These individuals could be potential sources for infection transmission, particularly with the absence of national precautionary steps or strict preventive measures at the borders (13). Given this situation, we aimed to assess the current state of the healthcare system in Yemen.

For demographics, the majority of respondents were male. This can be explained by the fact that males constitute the majority of Yemen's workforce (14). The limited number of consultants participating in this study could be justified by either they were too busy with patients, or they could have limited-time to access social networks. In addition, the number of consultants currently in Yemen has decreased significantly due to migration

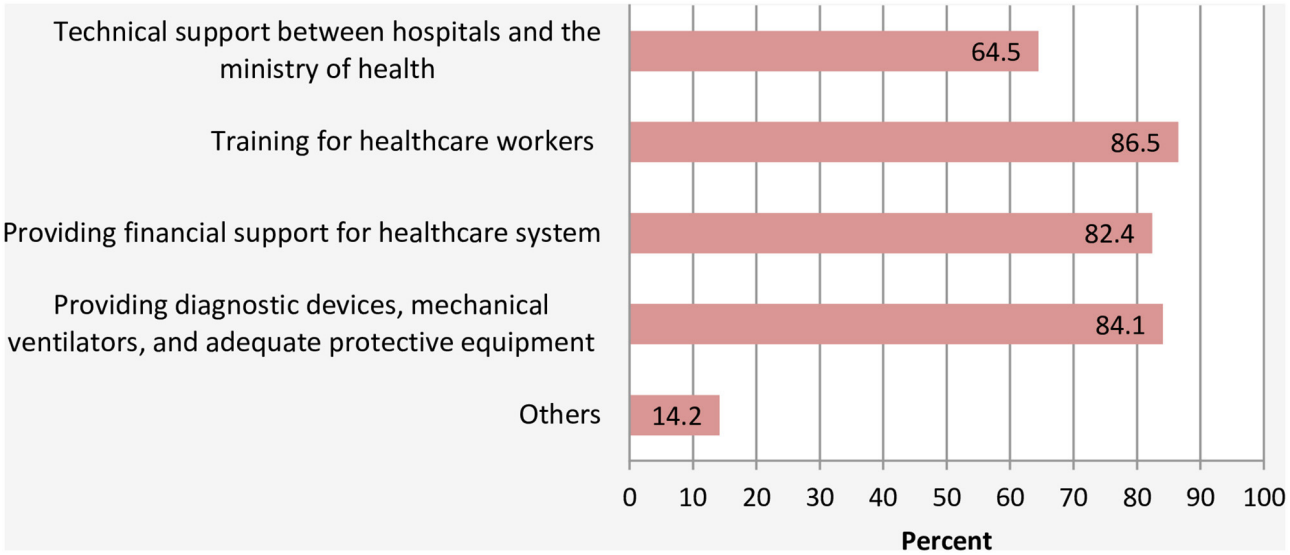


FIGURE 6 | Recommendations that should be made to face COVID-19.

abroad as a result of the ongoing war and conflict (15). Most of the healthcare workers were from hospitals in Sana'a, the capital of Yemen. This is justified by the fact that Sana'a is the largest city in Yemen and has the highest number of hospitals in the country (16).

The healthcare system capability and general preparedness to face COVID-19 was rated as very poor or poor by the majority of HCWs who participated in the study. This is consistent with international reports, which show that Yemen's healthcare system is fragile and has limited capacity to cope with public health emergencies (17). The country's infrastructure has been destroyed by more than 5 years of conflict. In this light, only <50% of HCFs are fully functioning, leaving a little capacity to respond to COVID-19 or other public health emergencies (11). Since 2015, there have been 142 attacks on hospitals and medical facilities across Yemen (18). By January 2017, four of the Médecins Sans Frontières (MSF) health facilities have been destroyed by airstrikes, resulting in casualties, including deaths, injuries, and ultimately forcing medical staff to leave the country (19).

Very poor availability of essential competencies such as mechanical ventilators, diagnostic devices, ICU rooms, beds, and isolation rooms, and lack of support to HCWs by local authorities is expected. Most of the facilities were left deserted by staff owing to security risks associated with working at those facilities. There is limited medicine, equipment, and personal protective equipment available, and only three testing sites for COVID-19, with a limited number of testing kits, are available in the entire country (Sana'a, Aden, and Al Mukalla). In addition to the war, several other reasons have contributed to pushing the healthcare system in Yemen to the brink of collapsing, including (1) declining public expenditure which due to deterioration in functions of public administration and contraction of country's economy (6); (2) the health system facilities were already overwhelmed by the outbreaks, cholera, and dengue (7, 20).

With regards to infection prevention and control measures, the majority of HCWs felt that their HCFs did not practice the simplest recommended preventive measures to minimize the spread of COVID-19. A significant proportion of HCFs did not adopt a policy for regulating the flow of people to the hospitals by decreasing the number of visitors and limiting the clinic and hospital visits to urgent and emergency cases. This huge gap in practicing these precautionary measures that require a no or low-cost for implementation could reflect the absence of emergency response and infection control plan within the hospitals prior to the outbreak of COVID-19 in Yemen. Social distancing has been identified as a crucial measure for COVID-19 containment and a vital step in slowing the spread of the novel coronavirus, not only in the community (21) but also within the hospitals (22). The risks of visitors with COVID-19 entering HCFs, queuing and staying in overcrowded waiting areas are very real, and the large outbreak of the Middle East respiratory syndrome coronavirus (MERS-CoV) infection in 2015 in South Korea gives a real example and provide us with valuable lessons of how dangerous a single patient exposure can be (23). Therefore, protecting the staff personnel and patients within the HCFs is paramount, and carrying out these proactive measures could play an essential role in the prevention and control of COVID-19.

For recommendations regarding the appropriate interventions to prompt the capabilities of the HCFs in facing COVID-19, the vast majority of respondents agreed that training of healthcare providers, providing them with the appropriate protective equipment, resources, and financial assistance, supporting the health information system for risk communication, and direct support with diagnostic devices and mechanical ventilators are needed. This majority consent

could reflect critical shortages of essential medial supply for prevention, control, and management of COVID-19 in Yemen. Other specific recommendations made by the HCWs, include directing the international financial support of COVID-19 containment to an independent committee, which was addressed by many healthcare providers. This reflects a lack of trust toward the local authorities. Others urged the government and international organizations to provide direct financial support to individuals who are being quarantined in the hospitals or being advised for self-isolation by the HCWs. This is very crucial due to the fact that 78% of the population is below the line of poverty, and the majority of them are daily wage workers (24). Thus, implementing such control measures without direct support could exacerbate their financial crisis.

LIMITATIONS

Our study has some limitations. First, the survey did not adequately cover all the hospitals in the country, with low responses were received from some governorates; thus, caution should be exercised in generalizing these findings. Also, due to time constrain and the current emergency state, the questionnaire was only face validated. Moreover, there was no official record-audit, and the data was a perspective of the HCWs. Thus, response bias cannot be rule out as participants may overestimate/underestimate the current capabilities of the HCFs they are working in. Despite these limitations, this is the first study investigating the capabilities and preparedness of Yemen's healthcare system for the COVID-19 pandemic. Also, the study used extensive sources of data, applied a rigorous methodology, and received responses from the main governmental and private healthcare facilities across the country. Finally, our findings are in line with the findings and field reports published by the UN and international organizations (4, 11).

CONCLUSION

The healthcare facilities in Yemen are unprepared and poorly equipped to cope with a COVID-19 outbreak. The majority of HCFs do not have enough ICU rooms, beds, isolation rooms, and there are huge deficits of essential medical supplies, testing capabilities, and protective equipment for personnel. Also, proactive measures for prevention and control of COVID-19 are not implemented or adequately enforced. With the current state of a fragile healthcare system, a widespread outbreak of COVID-19 in Yemen could result in devastating consequences. Support and interventions are urgently needed to face COVID-19 pandemic in Yemen.

Based on the current study findings, the following recommendations can be made:

1. Urgent interventions are required to provide PCR devices, diagnostic kits, mechanical ventilators, personnel protective equipment, and other essential medical supply to monitor, manage, and combat a COVID-19 outbreak in Yemen.
2. Training of more healthcare providers on infection control and emergency response is needed to combat the current COVID-19 outbreak.
3. Frontline healthcare personnel should be provided with the appropriate protective equipment to avoid any refusal to work at hospitals designed to receive individuals with COVID-19 infection.
4. Providing salaries on time and financial incentives for all healthcare workers are required to motivate them to engage in treatment and follow-up with COVID-19 patients.
5. Strict emergency plans and preventive measures are needed to be taken by the healthcare facilities.
6. We believe that the different authorities in Yemen should work together with the WHO on this dangerous situation by establishing a national emergency committee for the entire country, and a risk communication system for COVID-19 outbreak must be carried out between the different health authorities throughout the country with the help of international organizations, private, and NGOs-operated hospitals.
7. Building rapid response teams in each city for active surveillance, rapid detection, and management of suspected COVID-19 cases, as this will help in developing and implementing real-time preventive and control measures.
8. The government should be strict and proactive in enforcing the different measures for prevention, control of COVID-19 transmission.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the institutional review board committee at the University of Sciences and Technology, Sana'a, Yemen (ECA/UST189). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

RS designed the study conception. RS, MZ, FA-A, AK, and SSu analyzed the data and drafted the original manuscript. MK, SSa, RS, RA, MZ, and FA-A participated in data collection and manuscript editing. All authors reviewed and approved the final manuscript.

ACKNOWLEDGMENTS

The authors are grateful to Prof. Hilal Lashuel for his support and help in reviewing and editing this manuscript. We acknowledge the following HCWs for helping with data collection;

Faheem Al-Mughales, Dr. Kamal Al-Maqtri, Dr. Rasheed Al-Tamimi, Ibrahim Al-Samiee, Abdulateef Al-Mahmodi, and Yasser Saeed. Mohammed Hussein Zawiah would like to thank the institute of postgraduate studies of Universiti Sains Malaysia for providing fellowship during his course of doctoral studies.

REFERENCES

1. World Health Organization. *WHO Director-General's Statement on IHR Emergency Committee on Novel Coronavirus (2019-nCoV).* (2020). Available online at: https://www.who.int/dg/speeches/detail/who-director-general-s-statement-on-ihr-emergency-committee-on-novel-coronavirus-(2019-ncov) (accessed April 30, 2020).
2. World Health Organization. *WHO Director-General's Opening Remarks at the Media Briefing on COVID-19.* (2020). Available online at: https://www.who.int/dg/speeches/detail/who-director-general-s-opening-remarks-at-the-media-briefing-on-covid-19-$-$11-march-2020 (accessed April 30, 2020).
3. World Health Organization. *Coronavirus Disease 2019 (COVID-19): Situation Report, 101.* (2020). Available online at: https://www.who.int/docs/default-source/coronaviruse/situation-reports/20200411-sitrep-82-covid-19.pdf?sfvrsn=74a5d15_2 (accessed April 30, 2020).
4. UN Office of the Resident Coordinator and Humanitarian Coordinator for Yemen. *The Ministry of Health in Aden Confirms the First Case of COVID-19 in Yemen.* (2020). Available online at: https://reliefweb.int/sites/reliefweb.int/files/resources/HC%20Statement_COVID 19_First_Case.pdf (accessed April 30, 2020).
5. World Health Organization. *Yemen: Health Cluster Bulletin.* (2019). Available online at: https://www.who.int/health-cluster/countries/yemen/Yemen-Health-Cluster-Bulletin-Jan-2019.pdf (accessed May 1, 2020).
6. Qirbi N, Ismail SA. Health system functionality in a low-income country in the midst of conflict: the case of Yemen. *Health Policy Plan.* (2017) 32:911–22. doi: 10.1093/heapol/czx031
7. Camacho A, Bouhenia M, Alyusfi R, Alkohlani A, Naji MAM, de Radiguès X, et al. Cholera epidemic in Yemen, 2016–18: an analysis of surveillance data. *Lancet Global Health.* (2018) 6:e680–90. doi: 10.1016/S2214-109X(18)30230-4
8. Dureab F, Al-Awlaqi S, Jahn A. COVID-19 in Yemen: preparedness measures in a fragile state. *Lancet Public Health.* (2020) 5:e311. doi: 10.1016/S2468-2667(20)30101-8
9. Health system in Yemen close to collapse. *Bull World Health Organ.* (2015) 93:670–1. doi: 10.2471/BLT.15.021015
10. World Health Organization. *Yemen: Health Cluster Bulletin.* (2019). Available online at: https://www.who.int/health-cluster/countries/yemen/Yemen-Health-Cluster-Bulletin-December-2019.pdf?ua=1 (accessed May 1, 2020).
11. Nations U. *UN Offfice of the Redident Coordinator and Humanitarian Coordinator for Yemen Humanitarians Are Racing Against Time to Help Yemen Address COVID-19; The Odds Are Stacked Against Them.* (2020). Available online at: https://reliefweb.int/sites/reliefweb.int/files/resources/HC_Statement_on_COVID-19_23April2020.pdf?fbclid=IwAR3TTUcGb4r_QwRO6NA9GEdgMt2eQ3nj2ShkbCAx26T1gLr1LY-VdrUHoGY (accessed April 30, 2020).
12. World Health Organization. *Coronavirus Disease 2019 (COVID-19) Situation Report, 98.* (2020). Available online at: https://www.who.int/docs/default-source/coronaviruse/situation-reports/20200427-sitrep-98-covid-19.pdf?sfvrsn=90323472_4 (accessed April 28, 2020).
13. World Health Organization. *Coronavirus Disease 2019 (COVID-19) Situation Report – 80.* (2020). Available online at: https://www.who.int/docs/default-source/coronaviruse/situation-reports/20200409-sitrep-80-covid-19.pdf?sfvrsn=1b685d64_6 (accessed April 30, 2020).
14. International Labour Organisation. Regional Office for Arab States. *Yemen Labour Force Survey 2013-14.* (2015).
15. Al-Wesabi S. *Insecurity Drives Health Workers Out of Yemen.* World Health Organization (2016). Available online at: http://wwwwho~int/features/2016/yemen-health-insecurity/en (accessed May 1, 2020).
16. McMaster D, Bamashmus MA. Ophthalmic services during ongoing conflict: the eye health system in Yemen. *BMJ Global Health.* (2019) 4:e001743. doi: 10.1136/bmjgh-2019-001743
17. World Health Organization. *Yemen: Health System Profile, Key Health System Indicators.* (2013). Available online at: http://applications.emro.who.int/docs/Country_profile_2013_EN_15404.pdf?f (accessed April 29, 2020).
18. Uited Nations. *Report.* (2020). Available online at: https://reliefweb.int/report/yemen/second-attack-al-thawra-hospital-10-days-threatens-health-services-hundreds-thousands (accessed April 30, 2020).
19. Medecins sans Frontieres. *Yemen: Crisis Update.* (2017). Available online at: http://www.msf.org/en/article/yemen-crisis-update-january-2017 (accessed April 30, 2020).
20. Alghazali KA, Teoh B-T, Loong S-K, Sam S-S, Che-Mat-Seri N-A-A, Samsudin N-I, et al. Dengue outbreak during ongoing civil war, Taiz, Yemen. *Emerg Infect Dis.* (2019) 25:1397. doi: 10.3201/eid2507.180046
21. Wilder-Smith A, Freedman D. Isolation, quarantine, social distancing and community containment: pivotal role for old-style public health measures in the novel coronavirus (2019-nCoV) outbreak. *J Travel Med.* (2020) 27:taaa020. doi: 10.1093/jtm/taaa020
22. Wee LE, Conceicao EP, Sim XYJ, Aung MK, Tan KY, Wong HM, et al. Minimising intra-hospital transmission of COVID-19: the role of social distancing. *J Hosp Infect.* (2020) 105:113–5. doi: 10.1016/j.jhin.2020.04.016
23. Cho SY, Kang J-M, Ha YE, Park GE, Lee JY, Ko J-H, et al. MERS-CoV outbreak following a single patient exposure in an emergency room in South Korea: an epidemiological outbreak study. *Lancet.* (2016) 388:994–1001. doi: 10.1016/S0140-6736(16)30623-7
24. The World Bank. *The World Bank in Yemen.* (2019). Available online at: https://www.worldbank.org/en/country/yemen/overview (accessed April 30, 2020).

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Public Health

REVIEW
published: 28 July 2020
doi: 10.3389/fpubh.2020.00418

Racial and Gender-Based Differences in COVID-19

Jonathan Kopel[1], *Abhilash Perisetti*[2], *Ali Roghani*[1], *Muhammad Aziz*[3], *Mahesh Gajendran*[4] *and Hemant Goyal*[5]*

[1] Texas Tech University Health Sciences Center, Lubbock, TX, United States, [2] Department of Gastroenterology and Hepatology, University of Arkansas for Medical Sciences, Little Rock, AR, United States, [3] Department of Internal Medicine, The University of Toledo, Toledo, OH, United States, [4] Paul L. Foster School of Medicine, Texas Tech University Health Sciences Center El Paso, El Paso, TX, United States, [5] The Wright Center for Graduate Medical Education, Scranton, PA, United States

The novel coronavirus disease (COVID-19) has become a global health crisis since its first appearance in Wuhan, China. Current epidemiological studies suggest that COVID-19 affects older patients with multiple comorbidities, such as hypertension, obesity, and chronic lung diseases. The differences in the incidence and severity of COVID-19 are likely to be multifaceted, depending on various biological, social, and economical factors. Specifically, the socioeconomic differences and psychological impact of COVID-19 affecting males and females are essential in pandemic mitigation and preparedness. Previous clinical studies have shown that females are less susceptible to acquire viral infections and reduced cytokine production. Female patients have a higher macrophage and neutrophil activity as well as antibody production and response. Furthermore, *in-vivo* studies of the angiotensin-converting enzyme 2 (ACE2) showed higher expression in the kidneys of male than female patients, which may explain the differences in susceptibility and progression of COVID-19 between male and female patients. However, it remains unknown whether the expression of ACE2 differs in the lungs of male or female patients. Disparities in healthcare access and socioeconomic status between ethnic groups may influence COVID-19 rates. Ethnic groups often have higher levels of medical comorbidities and lower socioeconomic status, which may increase their risk of contracting COVID-19 through weak cell-mediated immunity. In this article, we examine the current literature on the gender and racial differences among COVID-19 patients and further examine the possible biological mechanisms underlying these differences.

Keywords: coronavirus, COVID-19, SARS-CoV-2, pandemic, sex, gender, race

OPEN ACCESS

Edited by:
Zisis Kozlakidis,
International Agency for Research on Cancer (IARC), France

Reviewed by:
Poornima Ramanan,
University of Colorado, United States
Jian-min Jin,
Capital Medical University, China

***Correspondence:**
Hemant Goyal
doc.hemant@yahoo.com

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Public Health

Received: *25 April 2020*
Accepted: *13 July 2020*
Published: *28 July 2020*

Citation:
Kopel J, Perisetti A, Roghani A, Aziz M, Gajendran M and Goyal H (2020) Racial and Gender-Based Differences in COVID-19. Front. Public Health 8:418. doi: 10.3389/fpubh.2020.00418

INTRODUCTION

The novel coronavirus disease (COVID-19) is caused by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2). The first reported case of COVID-19 was in December 2019 in Wuhan, China. The disease has continued to spread globally and was classified as a pandemic in March by the World Health Organization. Coronaviruses belong to a family of single-stranded RNA viruses, which cause several respiratory, gastrointestinal, hepatic, and neurologic diseases (1–3). Similar to the viruses causing Severe Acute Respiratory Syndrome (SARS) and Middle East Respiratory Syndrome (MERS), the SARS-CoV-2 is the seventh coronavirus (CoV) identified that can infect humans (2, 4, 5). Patients infected with SARS-CoV-2 have an incubation period of 3–14

days with a mean period of 5 days (1, 5, 6). The most common symptoms of COVID-19 include fever, cough, fatigue, and bleeding (2, 4, 5, 7–11). Other symptoms include taste changes, headache, abdominal pain, diarrhea, and gastrointestinal bleeding (2, 4, 5, 7–11). If left untreated, COVID-19 can lead to pneumonia, acute respiratory distress syndrome (ARDS), and acute respiratory failure resulting in death (1, 5, 6). The number one risk factor for severe disease is age with the severity increasing with the presence of comorbidities, such as heart and lung diseases (1, 5, 6). However, the effects of gender and ethnicity on SARS-CoV-2 infection rates and severity remain an area of active investigation.

The original clinical reports from China suggested that the COVID-19 virus infected both men and women equally; further studies suggested that sex differences exist in both mortality and infection susceptibility for SARS-CoV-2 (12, 13). From a socioeconomic perspective, school closures force more women with families to provide informal care for their immediate families, which limits women's work and economic opportunities for advancement (13). These differences are further compounded by the unique physical, sanitary, and security needs of women in quarantine conditions compared to men (13). Furthermore, data from the Centers for Disease Control (CDC) suggest that ethnic differences between COVID-19 patients may influence susceptibility and mortality. However, the mechanism for such differences remains mostly unknown (14). Another theory for these differences is related to differences in the expression of angiotensin-converting enzyme 2 (ACE2) receptors, which is the primary receptor for viral entry into the cells. In this article, we aim to elaborate on the effect of gender and ethnic backgrounds on COVID-19 infection and related mortality.

GENDER AND COVID-19

Studies With Male Predominance

The first cases of COVID-19 that occurred in China indicated the presence of gender differences (13). Initial reports estimated that 60% of COVID-19 patients were male (13). A study examining 799 patients in the Tongji Hospital in Wuhan, China found that of 113 COVID-19 deaths, 27% were female, and 73% were male (15). The authors concluded that the fatality rate was higher in men, possibly due to an increased prevalence of cardiopulmonary disease and smoking (15). Men were also more likely to develop heightened systemic inflammation, multi-organ dysfunction, and cardiac injury (15). A similar study of 54 deceased COVID-19 patients in South Korea showed that 61% of the patients were male.

A subsequent study examining 155 consecutive patients with confirmed COVID-19 in Zhongnan Hospital of Wuhan University found that 56% of the patients were male (16). A multivariate analysis performed in this study showed that male sex was a significant risk factor (OR: 2.206, 95% CI: 1.012–4.809) for prolonged (>14 days) COVID-19 symptoms (16). However, since 49% of these patients had chronic diseases, the authors believed that older male patients might have an increased risk for COVID-19, resulting in longer hospital stays and slow recovery (16). Similarly, another study showed that the male gender was a significant factor for COVID-19 infection on logistic regression analysis, with elderly male patients being at higher risk for the virus (17). Another study examined 46 deceased COVID-19 patients and found that men accounted for 67% of the fatalities (18). However, higher mortality in males could be a reflection of increased risk and prevalence of COVID-19 among male patients rather than being correlated with the male gender (18). A study examining 133 COVID-19 patients in Wuhan China, reported a similar male-predominance (58% male vs. 42% female) of COVID-19 infections (19). The study found that male patients were more likely than females (odds ratio: 3.24; 95% CI: 1.31–8.02) to shed the COVID-19 (20). Specifically, male patients continued to shed the COVID-19 virus for 18 days, while females shed the virus for 15.2 days (19).

Given the potential gender disparity, a recent study examined 4,880 COVID-19 patients who either had respiratory symptoms or close contact with a COVID-19 patients in Wuhan, China using quantitative RT-PCR (qRT-PCR) from nasopharyngeal samples. The study found no significant gender differences in the sample, which included 2,251 (46%) male and 2,629 (54%) female patients (17). However, 36.71% of females and 40.43% of males tested positive for COVID-19; furthermore, the positive rate of COVID-19 diagnosis using qRT-PCR was higher in males than females (17). Similarly, the positive rate for COVID-19 diagnosis also increased from 24.9 to 61.81% between younger and older patients (17). A recent study from COVID-19 patients in China showed that both the severity and mortality rates were worse among men than women (21). Specifically, men were over two times more likely to die from COVID-19 than women (21).

Studies With Female Predominance

Although the first reports in China showed a predominance of male COVID-19 patients, recent studies suggest that females may be at higher risk for COVID-19. The Korean Society of Infectious Diseases collected data on 4,212 COVID-19 patients, which showed that 37.7% were males while 62.3% were female (22). These results are in contrast to Chinese data, which estimated ∼51% of COVID-19 patients were male (22). The authors suggest the difference may reflect differences in social activities from different countries; in South Korea, the largest social age group are in their 20s. Furthermore, contact tracing of the COVID-19 outbreak in South Korea suggested that female practitioners of the Daegu religious sect may have contributed to the COVID-19 outbreak. Therefore, gender disparities in COVID-19 may reflect social and cultural differences between different countries (22). In a similar study in Qingdao City, China, examining 44 COVID-19 patients showed that 66% were female. The female predominance reported in this study was likely due to the small sample size during the early stages of the COVID-19 epidemic (23).

A larger study from the Zhejiang Province of China examined the gender distribution of COVID-19 patients in young and elderly patients (24). Young patient populations showed no significant difference in gender distribution (54% male 46% female). In contrast, the elderly (>60 years) COVID-19 patients were predominately female (43% male vs. 57% female) (24).

The differences in gender distribution are likely from increased medical comorbidities reported among elderly patients (older vs. younger groups: 55.15 vs. 21.93%) (24). Lastly, a multicenter European study examining 417 COVID-19 patients showed a higher proportion of COVID-19 patients were female (63%) than male (37%) (25). Interestingly, the study also found that female COVID-19 patients were more likely than males to be affected by olfactory and gustatory dysfunctions (25). It is unknown what biological process may be involved in female patients exhibiting a proportion of sensory dysfunction related to COVID-19 (25).

Studies With No Gender Predominance

Despite some studies showing gender differences in the incidence and case fatality rate in COVID-19 patients, a growing number of studies show no gender differences in SARS-CoV-2 infections. A study in Jiangsu Province, China, examined 80 patients with COVID-19 who found that men (49%) and women (51%) were equally affected (26). The authors of the study noted that the lack of gender differences could be related to the small sample size or the mode of transmission during the early stages of the pandemic (26). A similar study involving 135 patients, an equal distribution between men (53%) and women (47%) were noted with an average age of 47 years (27).

A study involving patients on a Japanese cruise ship found that among the 634 people who tested positive for COVID-19, 49% of cases were female and 51% male (20). The cases were from a total of 28 countries, including Japan (270 cases), the United States (88 cases), China (58 cases; including 30 from Hong Kong), Philippines (54 cases), Canada (51 cases), and Australia (49 cases) (20). Given the assortment of different ethnic groups in close proximity, this study suggests that COVID-19 infection rates may not depend on gender but may be reflective of underlying health status, comorbidities, and social factors within a given population.

Reproductive and Psychological Challenges of COVID-19 on Females

Despite the rapid spread of the disease, the COVID-19 outbreak has revealed unique challenges for both men and women. For pregnant women infected with COVID-19, there are reports of fetal distress, preterm delivery, and intrauterine virus transmission in the third trimester; however, the lack of information has produced much uncertainty concerning the overall health of the mother and fetus exposed to COVID-19 (28). Therefore, pregnant women are treated aggressively if exposed to COVID-19 and remain a vulnerable risk group to the COVID-19 virus (28). Furthermore, pregnant women face additional challenges with work, child-rearing, and maternal services, which further increase the risk of exposure to COVID-19 (28). Women may also have limited access to acute and emergency reproductive services forcing many women to travel long distances to safe medical facilities or have their child delivered at home in developing countries (28). In addition, cesarean sections and abortion care are also limited due to staff shortages and increased risk of COVID-19 infections in surgical wards (28). In poorer countries, these limitations are further compounded by the limited access to routine clinical care for contraceptive counseling or other reproductive health services (28).

As a result, the females are associated with a more significant psychological impact leading to higher levels of stress, anxiety, and depression (29, 30). A study following the outbreak in Wuhan, China, found that the level of post-traumatic stress syndrome (PTSS) was 7% in women (21.9%) who reported higher negative alterations in mood and hyper-arousal than men (14.6%) (30). A survey conducted by the Kaiser Family Foundation in the US telephone interviewed 1,126 adults to determine the differences in men and women responding to the COVID-19 pandemic (31). The survey found that more women than men worry that they or someone in their family will get sick from the coronavirus (68 vs. 56%) or are concerned about losing income due to a workplace closure or reduced hours (50 vs. 42%) (31). In addition, more women compared to men worry that they would put themselves at risk of exposure to coronavirus because they cannot afford to stay home and miss work (39 and 31%). Women also reported having more part-time jobs than men (13 vs. 9%) and worried more than men about whether they would be able to afford testing or treatment for coronavirus if they need it (40 vs. 31%) (31). Given the increased stress reported in this survey, women were also asked questions about their mental health. The survey found that more women (16%) than men (11%) felt that worry or stress related to COVID-19 would have a significantly negative impact on their mental health. Furthermore, four in 10 women (36%) and three in 10 men (27%) felt worried or stressed about how coronavirus has had some impact on their mental health (31).

Despite the emotional challenges faced by women, women reported taking more precautions to reduce their exposure and spread of COVID-19 compared to men. The survey found that more women compared to men say they decided not to travel or changed travel plans (47 vs. 37%), or reported canceling plans to attend large gatherings (43 vs. 36%). Furthermore, women were more likely than men to stock food, household supplies, or prescription medications (39 vs. 30%), and planned to stay at home instead of going to work, school, or other regular activities (30 vs. 22%). In this respect, women may act as a safety net and an essential component for maintaining social distancing with their families and society at large by balancing several responsibilities. Studies show that women play an essential role in the stages of public health management, including planning, decision-making, and emergency response systems (32). Furthermore, women are the primary caregiver for the young, the elderly, and sick in most households and healthcare facilities (32). Despite this, women remain under-represented in most political and healthcare organizations (32). Specifically, political and healthcare organizations with a higher representation of women had a lower number of COVID-19 cases and hospitalizations (32). Without a long-term policy response, including more female representation, these issues will persist long after the COVID-19 pandemic has passed.

RACE AND COVID-19 INFECTION

Current epidemiological data on COVID-19 suggests that minority groups may also be more susceptible to COVID-19 infections (14). A study conducted by the CDC and the COVID-19–Associated Hospitalization Surveillance Network during March 2020 examined 1,482 hospitalized patients across 14 states in the US (14). The study found that 54 and 46% of hospitalized patients were male and female, respectively (14). Furthermore, the COVID-19-associated hospitalization rates were higher among males than among females (5.1 vs. 4.1 per 100,000 population) (14). Furthermore, CDC and COVID-19–Associated Hospitalization Surveillance Network examined 580 of the 1,482 COVID-19 patients with race/ethnicity data and found that 45.0% were Caucasian, 33% African-American, 8% Hispanic, 5% Asian, <1% American Indian/Alaskan Native, and 7.9% were of other or unknown race (14). These results are impressive since 33% of hospitalized patients were African American even though they constitute 13% of the United States (US) population. In contrast, 8% of hospitalized patients were Hispanics, who make up 18% of the US population, and 45% of hospitalized COVID-19 patients were Caucasian, who make 76% of the US population.

However, the study noted racial distributions of hospitalized COVID-19 varied state by state depending on the population of interest (14). Furthermore, minority populations, such as African Americans, are more likely to have diabetes, hypertension, obesity, asthma, and heart disease, which increases their risk of contracting COVID-19. The CDC and COVID-19–Associated Hospitalization Surveillance Network reported that 89% of hospitalized COVID-19 patients had some form of a pre-existing condition. Specifically, 50% of hospitalized patients had hypertension, 48% had obesity, 35% had chronic lung diseases, 28% had diabetes mellitus, and 28% had cardiovascular disease (14). A previous study examining cytomegalovirus (CMV) among different socioeconomic groups in the US found that a reduced socioeconomic status was associated with a more inferior cell-mediated immunity (33). The study suggested that patients with a lower socioeconomic status predispose them to reduced access to medical care, multiple comorbidities, poor diet, and life stressors that could weaken their immune system.

Given that many minority populations have higher proportions of patients with low socioeconomic status, this may be a contributing factor to the higher prevalence of COVID-19 infections (33–35). Current COVID-19 guidelines encourage clinicians to perform preventive measures, such as social distancing, respiratory hygiene, and wearing face coverings in public settings, to protect older adults and persons with underlying medical conditions (14). There may exist some racial disparity in COVID-19 infections, given the data published on SARS infections (36). A previous study examining polymorphisms in mannose-binding lectin (MBL) genes found that MBL gene polymorphisms were associated with increased susceptibility to SARS-CoV infection (36). Further data collection and research are needed to determine if a racial disparity exists with COVID-19 and which socioeconomic and biological factors are involved.

Although few studies have examined the biological mechanisms underlying ethnic differences of COVID-19 infection susceptibility, a recent study noted that ACE2 expression could vary among Asians (significantly higher) compared to African Americans and Caucasians (37). Though this might explore susceptibility patterns among different ethnicities, larger studies are needed to validate these. Therefore, it remains open whether gender or ethnic differences exist with the expression of ACE2, and ultimately, the pathogenesis of COVID-19.

The major limitation in determining whether gender or ethnic differences exist for COVID-19 patients is the lack of pre-clinical and clinical studies. The significant studies for documenting the epidemiological data for COVID-19 patients were from Wuhan, China. Only two studies included data outside of Wuhan, China, and these data sets were located in Asian countries (Japan and Korea). Therefore, there is a significant bias in the ethnic group represented in these samples. Besides, the limited number of patients diagnosed with COVID-19 may be much higher than reported, given the lack of systematic testing during the initial stages of the outbreak. Furthermore, patients presenting with mild symptoms of cough, fever, and headache may have been misdiagnosed with influenza rather than COVID-19. Given the rapid changes in this pandemic, the data on infection rate, morbidity, and mortality between male and female COVID-19 patients will likely improve with larger sample sizes and greater distribution of age and ethnic backgrounds.

PUTATIVE MECHANISMS FOR GENDER DIFFERENCES AMONG COVID-19 PATIENTS

Although the precise mechanism of gender differences in COVID-19 remains unknown, previous studies provide insights into possible mechanisms. Previous studies have shown females have increased resistance to viral, bacterial, fungal, and parasitic organisms than males (38). Specifically, females are less susceptible to microbial infections by mounting a higher innate immune response than males (39, 40). Women produce a higher expression of the inflammatory and cytotoxic proteins, including interferon-g (IFN-g), lymphotoxin b (LTb), granzyme A (GZMA), interleukin-12 receptor b2 (IL12Rb2), and granulysin (GNLY) (39). Furthermore, female patients are less likely to produce extreme immune responses to bacterial or viral infections than male patients leading to sepsis. A study examining gender differences in sepsis patients found that male patients have higher circulating levels of TNF-α than female patients, which are correlated with a worse prognosis (41). Female sepsis patients were protected from sepsis due to the increased production of the immunosuppressive cytokine IL-10 (41). Specifically, female patients produce lower levels of inflammatory mediators and increase the production of immunosuppressive molecules to reduce systemic inflammation. The protection of females to microbial and viral affections is attributed to the protection provided by the X chromosome and sex hormones, which modulate the innate and adaptive immunity (38). In

contrast, males are at higher risk of developing cancers as opposed to females who have a higher risk of autoimmune diseases (38).

Hormonal Effect

Estrogen has been well-documented as a positive stimulator of the immune response, particularly with increasing the activity and proliferation of T-cells (42). Estrogen suppresses the immune system by reducing the production of IL-1β, IL-6, and TNF by monocytes (43). Estrogen also reduces the expression of nitric oxide synthase, which impairs chemotaxis of neutrophils (44, 45). Furthermore, estrogen increases the expression of Toll-like Receptor 4 (TLR4) and CD14 expression on macrophages and the differentiation and activation of dendritic cells (38). Lastly, estrogen increases humoral responses from B lymphocytes producing more antibodies in females than males through enhancing IgG and IgM antibodies (38).

Testosterone shows several immunosuppressive functions by reducing cytokine production and proliferation of lymphocytes (38). It increases neutrophil activation in non-infectious states while reducing the expression of TLR4 (38). It reduces IgM and IgG production directly and by reducing the production of IL-6 by monocytes (38). As a result, men with higher testosterone levels have been reported to have lower titers of antibodies after vaccination compared to women who have lower testosterone levels (38). Therefore, the levels of testosterone and estrogen between men and women could predispose individuals to different levels of severity in COVID-19 symptoms.

Infection Susceptibility

Women are less susceptible to viral infections than men due to their mounting of more robust immune responses. A study comparing HIV-1 infections in men and women showed that untreated HIV-1 infected women had 40% less circulating viral RNA and greater activation in $CD8^+$ T cells than men (46, 47). Similar studies with Hepatitis B and C viruses, HIV, Hantavirus, West Nile Virus infections, and influenza viruses showed that men were more susceptible than females to viral infections (48). However, the study also found that the hyperactive response in female immune systems to viral infections may increase symptom severity and pathological effects than observed in male patients (49). The authors hypothesized that effect could result from an increase in the production of cytokines, chemokines, and interferons in females than males (50). Following the elimination of the viral infection, females maintain elevated immune responses that can increase the risk of post-infection complications compared to men (50). Given these disparities in viral symptoms, female patients infected with COVID-19 may experience more long-term complications than men. Further investigation into the immune response to COVID-19 between male and female patients will be needed.

Gender Differences in ACE2

It remains uncertain whether there are gender differences in the expression of ACE2 receptor, which is the protein involved in the first step of viral entry for COVID-19 (51). ACE-2 is a type-1 transmembrane metallo-carboxypeptidase that regulates blood pressure through the renin-angiotensin systems (RAS) (52). The ACE-2 degrades Angiotensin II to generate Angiotensin 1–7, thereby negatively regulating RAS (53, 54). Although ACE-2 is expressed mostly in the vascular endothelial cells, the renal tubular epithelium, and in Leydig cells in the testes, ACE-2 has also been detected in several other organs and tissues, such as the nasopharynx, lung, stomach, small intestine, colon, lymph nodes, thymus, bone marrow, spleen, liver, kidney, and brain (55, 56).

In vivo studies suggest that ACE2 expression in the kidney is higher in males than females due to the presence of testosterone and estrogen regulatory activities on post-transcriptional and post-translational mechanisms on ACE2 expression (57, 58). However, the same study examining rat lung ACE-2 expression also showed a decreased expression of ACE-2 with increasing rat age (59). Furthermore, there was no difference observed in the ACE2 expression in the heart and lungs of both rats and mice (60, 61). Interestingly, the expression of ACE2 changes through the life-span of female rats through fluctuations in estradiol (E_2); in particular, the expression of ACE2 increases significantly during pregnancy (62). Together, these studies suggest a combination of social and biological differences (e.g., ACE-2 expression) in pregnant female patients may predispose them to COVID19. Medical comorbidities with ACE-2 expression may also explain the increased susceptibility of pregnant female patients to COVID-19. However, further histological and pathology studies are needed to examine the influence of age and gender on the expression of lung ACE-2 and the risk of patients for COVID-19 infection.

Currently, smaller sample sizes or cases are being reported in the literature to assess the gender and ethnic differences of COVID-19 patients (63). Furthermore, the lack of systematic testing across the world limits the accuracy of epidemiological data for the distribution of COVID-19 patients. Second, the morbidity and mortality rates for gender and ethnic groups should be stratified in future epidemiological studies on COVID-19 to assess the differences in healthcare interventions and outcomes for each group (63). Third, there needs to be an increase in scientific and pathological studies assessing the expression and activity of ACE-2 to identify which patient populations are most at risk. Currently, there have been few studies investigating ACE-2 expression in COVID-19 patients and whether ACE-2 inhibitors may provide therapeutic benefit. Further genetic and biomedical studies are needed to determine if other biomarkers exist for evaluating susceptible populations and guiding patient management. This information would provide clinicians a broader perspective of the biological, social, and economic factors influencing the susceptibility and management of COVID-19 patients.

GAPS IN KNOWLEDGE

There is a greater need to focus on the unique challenges male patients face with the COVID-19 pandemic (64). A recent study showed that there was a 29% reduction in men seeking medical treatment for ST-segment elevation myocardial infarction during the COVID-19 pandemic; in contrast, there

was no change in women hospitalized (64). It is believed that the association of cardiovascular illness among men with COVID-19 may cause male patients to be reluctant in seeking medical attention for cardiovascular illness (64). Furthermore, the co-existence of COVID-19 symptoms may mask the symptoms of a heart attack among male patients (64). Male patients are also known to have a higher treatment-seeking threshold due to the prevalence of male stoicism and self-reliance (64). Therefore, physicians may be required to investigate other medical conditions in addition to COVID-19 to avoid under-diagnosing potential devastating medical conditions, such as heart disease, among male COVID-19 patients.

The current epidemiological data suggests that men are more affected than women by the COVID-19 virus (65). However, these studies are limited by location, sample size, and other potential biases in the population examined. Different clinical studies have given conflicting reports on the male or female predominance of COVID-19 infections. This discrepancy is likely due to the lack of large-scale epidemiological studies, socioeconomic disparities, or other confounders on the prevalence of pre-existing conditions in different countries. Recent epidemiological data from 38 countries showed a male predominance in COVID-19 infections, which increased in older age demographics. Furthermore, the case fatality rate was 1.7 times higher in men than females (65). The authors of the study suggested that differences in sex hormones, sex chromosomes, genetic polymorphisms, and epigenetic modifications between males and females may impact immune responses (65). Specifically, the authors reported that the ACE-2 receptor is located on the X chromosome and is down-regulated by increased estrogen levels (65). However, the expression of ACE-2 seems to increase with age and with the menstrual cycle of female mice. Therefore, it is possible that the increased expression of ACE-2 expression in elderly and pregnant patients may increase their risk for COVID-19. In general, the increased estrogen in females reduces their chance of viral infections from increase macrophage/neutrophil/dendritic cell activity, humoral response, and T-cell function compared to males. It is believed that the increased immune response in females reduces their susceptibility to COVID-19. The biological mechanism behind these differences also needs further elucidation.

CONCLUSION

As the COVID-19 pandemic spreads, the differences between male and female mortality and infectivity remains an area of active investigation. The current literature suggests that men tend to have a higher risk of severe infection and mortality related to COVID-19. It is believed that elevated estrogen levels in female COVID-19 patients may reduce the severity and mortality of COVID-19 deaths through an elevation in the innate and humoral response. Furthermore, pre-clinical studies suggest that ACE-2 expression may increase the susceptibility of COVID-19 in pregnant patients. Similarly, the severity and mortality with COVID-19 differ between different ethnic groups. Although genetic polymorphisms associated with COVID-19 susceptibility among ethnic groups remains unknown, the increase in medical comorbidities and lack of access to care are significant contributors to increased COVID-19 mortality in these communities. As the pandemic continues to spread, African American and Hispanic communities have shown increased rates of infection and hospitalization compared to Caucasian populations. These differences are likely due to a higher prevalence of diabetes, hypertension, obesity, asthma, and heart disease in minority groups.

Further research is required to understand the molecular and pathophysiological mechanisms underlying these ethnic disparities in COVID-19 infection and severity. It remains unknown though whether genetic polymorphisms in ACE-2 expression or other genes may be involved in the gender or ethnic disparities to COVID-19 infection. Furthermore, the role of ACE-2 expression in male and female COVID-19 patients remains unknown. Pre-clinical studies show no difference in ACE-2 expression between male and female murine models. Additional studies are needed to evaluate whether expression levels of ACE-2 in the lung correlated with the severity or susceptibility of COVID-19 in human subjects. In the meantime, policies for reducing the spread of COVID-19 should take into consideration the unique challenges among men and women, including social, emotional, physical, and economic security.

AUTHOR CONTRIBUTIONS

AP and HG: conception and design. JK: first draft. All authors: literature review, critical revision and editing, and final approval.

REFERENCES

1. Wu D, Wu T, Liu Q, Yang Z. The SARS-CoV-2 outbreak: what we know. *Int J Infect Dis.* (2020) 94:44–8. doi: 10.1016/j.ijid.2020.03.004
2. Rothan HA, Byrareddy SN. The epidemiology and pathogenesis of coronavirus disease (COVID-19) outbreak. *J Autoimmun.* (2020) 2020:102433. doi: 10.1016/j.jaut.2020.102433
3. Kopel J, Perisetti A, Gajendran M, Boregowda U, Goyal H. Clinical insights into the gastrointestinal manifestations of COVID-19. *Digest Dis Sci.* (2020) 65:1932–9. doi: 10.1007/s10620-020-06362-8
4. Lake MA. What we know so far: COVID-19 current clinical knowledge and research. *Clin Med (Lond).* (2020) 20:124–7. doi: 10.7861/clinmed.2019-coron
5. Singhal T. A review of coronavirus disease-2019 (COVID-19). *Indian J Pediatr.* (2020) 87:281–6. doi: 10.1007/s12098-020-03263-6
6. Velavan TP, Meyer CG. The COVID-19 epidemic. *Trop Med Int Health.* (2020) 25:278–80. doi: 10.1111/tmi.13383
7. Perisetti A, Gajendran M, Boregowda U, Bansal P, Goyal H. COVID-19 and gastrointestinal endoscopies: current insights and emergent strategies. *Digest Endosc.* (2020) 32:715–22. doi: 10.1111/den.13693
8. Gadiparthi C, Perisetti A, Sayana H, Tharian B, Inamdar S, Korman A. Gastrointestinal bleeding in patients with severe SARS-CoV-2. *Am J Gastroenterol.* (2020). doi: 10.14309/ajg.0000000000000719. [Epub ahead of print].

9. Perisetti A, Gajendran M, Goyal H. Putative mechanisms of diarrhea in COVID-19. *Clin Gastroenterol Hepatol.* (2020). doi: 10.1016/j.cgh.2020.06.008. [Epub ahead of print].
10. Aziz M, Perisetti A, Lee-Smith WM, Gajendran M, Bansal P, Goyal H. Taste changes (Dysgeusia) in COVID-19: a systematic review and metaanalysis. *Gastroenterology.* (2020). doi: 10.1053/j.gastro.2020.05.003. [Epub ahead of print].
11. Ramachandran P, Onukogu I, Ghanta S, Gajendran M, Perisetti A, Goyal H, et al. Gastrointestinal symptoms and outcomes in hospitalized COVID-19 patients. *Digest Dis.* (2020). doi: 10.1159/000509774. [Epub ahead of print].
12. Wenham C, Smith J, Morgan R. COVID-19: the gendered impacts of the outbreak. *Lancet.* (2020) 395:846–8. doi: 10.1016/S0140-6736(20)30526-2
13. Li L, Huang T, Wang Y, Wang Z, Liang Y, Huang T, et al. COVID-19 patients' clinical characteristics, discharge rate, and fatality rate of meta-analysis. *J Med Virol.* 92:577–83. doi: 10.1002/jmv.25757
14. Garg S, Kim L, Whitaker M, O'Halloran A, Cummings C, Holstein R, et al. Hospitalization rates and characteristics of patients hospitalized with laboratory-confirmed coronavirus disease 2019—COVID-NET, 14 states, March 1–30, 2020. *MMWR Morb Mortal Weekly Rep.* (2020) 69:458–64. doi: 10.15585/mmwr.mm6915e3
15. Clinical characteristics of 113 deceased patients with coronavirus disease 2019: retrospective study. *BMJ.* (2020) 368:m1295. doi: 10.1136/bmj.m1091
16. Mo P, Xing Y, Xiao Y, Deng L, Zhao Q, Wang H, et al. Clinical characteristics of refractory COVID-19 pneumonia in Wuhan, China. *Clin Infect Dis.* (2020). doi: 10.1093/cid/ciaa270. [Epub ahead of print].
17. Liu R, Han H, Liu F, Lv Z, Wu K, Liu Y, et al. Positive rate of RT-PCR detection of SARS-CoV-2 infection in 4880 cases from one hospital in Wuhan, China, from Jan to Feb 2020. *Clin Chim Acta.* (2020) 505:172–5. doi: 10.1016/j.cca.2020.03.009
18. Leung C. Clinical features of deaths in the novel coronavirus epidemic in China. *Rev Med Virol.* (2020) 30:e2103. doi: 10.1002/rmv.2103
19. Xu K, Chen Y, Yuan J, Yi P, Ding C, Wu W, et al. Factors associated with prolonged viral RNA shedding in patients with COVID-19. *Clin Infect Dis.* (2020). doi: 10.1093/cid/ciaa351. [Epub ahead of print].
20. Mizumoto K, Kagaya K, Zarebski A, Chowell G. Estimating the asymptomatic proportion of coronavirus disease 2019 (COVID-19) cases on board the Diamond Princess cruise ship, Yokohama, Japan, 2020. *Eurosurveillance.* (2020) 25:2000180. doi: 10.2807/1560-7917.ES.2020.25.10.2000180
21. Jin J-M, Bai P, He W, Wu F, Liu X-F, Han D-M, et al. Gender differences in patients with COVID-19: focus on severity and mortality. *Front Public Health.* (2020) 8:152. doi: 10.3389/fpubh.2020.00152
22. Korean Society of Infectious Diseases, Korean Society of Pediatric Infectious Diseases, Korean Society of Epidemiology, Korean Society for Antimicrobial Therapy, Korean Society for Healthcare-associated Infection Control and Prevention, Korea Centers for Disease Control and Prevention. Report on the epidemiological features of coronavirus disease 2019 (COVID-19) outbreak in the Republic of Korea from January 19 to March 2, 2020. *J Korean Med Sci.* (2020) 35:e112. doi: 10.3346/jkms.2020.35.e112
23. Jia J, Hu X, Yang F, Song X, Dong L, Zhang J, et al. Epidemiological characteristics on the clustering nature of COVID-19 in Qingdao City, 2020: a descriptive analysis. *Disaster Med Public Health Prep.* (2020). doi: 10.1017/dmp.2020.59. [Epub ahead of print].
24. Lian J, Jin X, Hao S, Cai H, Zhang S, Zheng L, et al. Analysis of epidemiological and clinical features in older patients with corona virus disease 2019 (COVID-19) out of Wuhan. *Clin Infect Dis.* (2020) 382:ciaa242. doi: 10.1093/cid/ciaa242
25. Lechien JR, Chiesa-Estomba CM, De Siati DR, Horoi M, Le Bon SD, Rodriguez A, et al. Olfactory and gustatory dysfunctions as a clinical presentation of mild-to-moderate forms of the coronavirus disease (COVID-19): a multicenter European study. *Eur Archiv Otorhinolaryngol.* (2020) 277:2251–61. doi: 10.1007/s00405-020-05965-1
26. Wu J, Liu J, Zhao X, Liu C, Wang W, Wang D, et al. Clinical characteristics of imported cases of coronavirus disease 2019 (COVID-19) in Jiangsu province: a multicenter descriptive study. *Clin Infect Dis.* (2020). doi: 10.1093/cid/ciaa199. [Epub ahead of print].
27. Wan S, Xiang Y, Fang W, Zheng Y, Li B, Hu Y, et al. Clinical features and treatment of COVID-19 patients in northeast Chongqing. *J Med Virol.* (2020) 92:797–806. doi: 10.1002/jmv.25783
28. Hussein J. COVID-19: What implications for sexual and reproductive health and rights globally? *Sexual Reprod Health Matters.* (2020) 28:1746065. doi: 10.1080/26410397.2020.1746065
29. Wang C, Pan R, Wan X, Tan Y, Xu L, Ho CS, et al. Immediate psychological responses and associated factors during the initial stage of the 2019 coronavirus disease (COVID-19) epidemic among the general population in China. *Int J Environ Res Public Health.* (2020) 17:1729. doi: 10.3390/ijerph17051729
30. Liu N, Zhang F, Wei C, Jia Y, Shang Z, Sun L, et al. Prevalence and predictors of PTSS during COVID-19 outbreak in China hardest-hit areas: gender differences matter. *Psychiatry Res.* (2020) 287:112921. doi: 10.1016/j.psychres.2020.112921
31. Frederiksen B, Gomez I, Salganicoff A, Ranji U. *Coronavirus: A Look at Gender Differences in Awareness and Actions*. Kaiser Family Foundation-Women's Health Policy (2020).
32. Leung TY, Sharma P, Adithipyangkul P, Hosie P. Gender equity and public health outcomes: the COVID-19 experience. *J Bus Res.* (2020) 116:193–8. doi: 10.1016/j.jbusres.2020.05.031
33. Dowd JB, Aiello AE. Socioeconomic differentials in immune response. *Epidemiology.* (2009) 20:902–8. doi: 10.1097/EDE.0b013e3181bb5302
34. Adler NE. Socioeconomic inequalities in health. No easy solution. *J Am Med Assoc.* (1993) 269:3140–5. doi: 10.1001/jama.269.24.3140
35. Steptoe A, Shamaei-Tousi A, Gylfe A, Henderson B, Bergstrom S, Marmot M. Socioeconomic status, pathogen burden and cardiovascular disease risk. *Heart.* (2006) 93:1567–70. doi: 10.1136/hrt.2006.113993
36. Zhang H, Zhou G, Zhi L, Yang H, Zhai Y, Dong X, et al. Association between Mannose-binding lectin gene polymorphisms and susceptibility to severe acute respiratory syndrome coronavirus infection. *J Infect Dis.* (2005) 192:1355–61. doi: 10.1086/491479
37. Zhao Y, Zhao Z, Wang Y, Zhou Y, Ma Y, Zuo W. Single-cell RNA expression profiling of ACE2, the putative receptor of Wuhan 2019-nCov. *Cold Spring Harbor Lab.* (2020). doi: 10.1101/2020.01.26.919985. [Epub ahead of print].
38. Jaillon S, Berthenet K, Garlanda C. Sexual dimorphism in innate immunity. *Clin Rev Allergy Immunol.* (2017) 56:308–21. doi: 10.1007/s12016-017-8648-x
39. Hewagama A, Patel D, Yarlagadda S, Strickland FM, Richardson BC. Stronger inflammatory/cytotoxic T-cell response in women identified by microarray analysis. *Genes Immunity.* (2009) 10:509–16. doi: 10.1038/gene.2009.12
40. Marriott I, Huet-Hudson YM. Sexual dimorphism in innate immune responses to infectious organisms. *Immunol Res.* (2006) 34:177–92. doi: 10.1385/IR:34:3:177
41. Schröder J. Gender differences in human sepsis. *Archiv Surg.* (1998) 133:1200. doi: 10.1001/archsurg.133.11.1200
42. Mohammad I, Starskaia I, Nagy T, Guo J, Yatkin E, Väänänen K, et al. Estrogen receptor α contributes to T cell–mediated autoimmune inflammation by promoting T cell activation and proliferation. *Sci Signal.* (2018) 11:eaap9415. doi: 10.1126/scisignal.aap9415
43. Kramer PR, Winger V, Kramer SF. 17β-Estradiol utilizes the estrogen receptor to regulate CD16 expression in monocytes. *Mol Cell Endocrinol.* (2007) 279:16–25. doi: 10.1016/j.mce.2007.08.014
44. GarcÍa-Durán M, de Frutos T, DÍaz-Recasens Jn, GarcÍa-Gálvez G, Jiménez A, Montón M, et al. Estrogen stimulates neuronal nitric oxide synthase protein expression in human neutrophils. *Circ Res.* (1999) 85:1020–6. doi: 10.1161/01.RES.85.11.1020
45. Miyagi M, Aoyama H, Morishita M, Iwamoto Y. Effects of sex hormones on chemotaxis of human peripheral polymorphonuclear leukocytes and monocytes. *J Periodontol.* (1992) 63:28–32. doi: 10.1902/jop.1992.63.1.28
46. Ghosh S, Klein RS. Sex drives dimorphic immune responses to viral infections. *J Immunol.* (2017) 198:1782–90. doi: 10.4049/jimmunol.1601166
47. Sterling TR, Vlahov D, Astemborski J, Hoover DR, Margolick JB, Quinn TC. Initial plasma HIV-1 RNA levels and progression to AIDS in women and men. *N Engl J Med.* (2001) 344:720–5. doi: 10.1056/NEJM200103083441003
48. Klein SL. The effects of hormones on sex differences in infection: from genes to behavior. *Neurosci Biobehav Rev.* (2000) 24:627–38. doi: 10.1016/S0149-7634(00)00027-0
49. Oertelt-Prigione S. The influence of sex and gender on the immune response. *Autoimmun Rev.* (2012) 11:A479–85. doi: 10.1016/j.autrev.2011.11.022

50. Klein SL. Sex influences immune responses to viruses, and efficacy of prophylaxis and treatments for viral diseases. *Bioessays.* (2012) 34:1050–9. doi: 10.1002/bies.201200099
51. Xiao F, Tang M, Zheng X, Liu Y, Li X, Shan H. Evidence for gastrointestinal infection of SARS-CoV-2. *Gastroenterology.* (2020) 158:1831–3.e3. doi: 10.1101/2020.02.17.20023721
52. Riordan JF. Angiotensin-I-converting enzyme and its relatives. *Genome Biol.* (2003) 4:225. doi: 10.1186/gb-2003-4-8-225
53. Kuba K, Imai Y, Ohto-Nakanishi T, Penninger JM. Trilogy of ACE2: a peptidase in the renin–angiotensin system, a SARS receptor, and a partner for amino acid transporters. *Pharmacol Ther.* (2010) 128:119–28. doi: 10.1016/j.pharmthera.2010.06.003
54. Tikellis C, Thomas MC. Angiotensin-converting enzyme 2 (ACE2) is a key modulator of the renin angiotensin system in health and disease. *Int J Pept.* (2012) 2012:1–8. doi: 10.1155/2012/256294
55. Ksiazek TG, Erdman D, Goldsmith CS, Zaki SR, Peret T, Emery S, et al. A novel coronavirus associated with severe acute respiratory syndrome. *N Engl J Med.* (2003) 348:1953–66. doi: 10.1056/NEJMoa030781
56. Harmer D, Gilbert M, Borman R, Clark KL. Quantitative mRNA expression profiling of ACE 2, a novel homologue of angiotensin converting enzyme. *FEBS Lett.* (2002) 532:107–10. doi: 10.1016/S0014-5793(02)03640-2
57. Liu J, Ji H, Zheng W, Wu X, Zhu JJ, Arnold AP, et al. Sex differences in renal angiotensin converting enzyme 2 (ACE2) activity are 17β-oestradiol-dependent and sex chromosome-independent. *Biol Sex Differ.* (2010) 1:6. doi: 10.1186/2042-6410-1-6
58. Sampson AK, Moritz KM, Denton KM. Postnatal ontogeny of angiotensin receptors and ACE2 in male and female rats. *Gender Med.* (2012) 9:21–32. doi: 10.1016/j.genm.2011.12.003
59. Xudong X, Junzhu C, Xingxiang W, Furong Z, Yanrong L. Age- and gender-related difference of ACE2 expression in rat lung. *Life Sci.* (2006) 78:2166–71. doi: 10.1016/j.lfs.2005.09.038
60. Brosnihan KB, Hodgin JB, Smithies O, Maeda N, Gallagher P. Tissue-specific regulation of ACE/ACE2 and AT1/AT2 receptor gene expression by oestrogen in apolipoprotein E/oestrogen receptor-α knock-out mice. *Exp Physiol.* (2008) 93:658–64. doi: 10.1113/expphysiol.2007.041806
61. Pendergrass KD, Pirro NT, Westwood BM, Ferrario CM, Brosnihan KB, Chappell MC. Sex differences in circulating and renal angiotensins of hypertensive mRen().Lewis but not normotensive Lewis rats. *Am J Physiol Heart Circ Physiol.* (2008) 295:H10–20. doi: 10.1152/ajpheart.01277.2007
62. Brosnihan KB, Neves LAA, Joyner J, Averill DB, Chappell MC, Sarao R, et al. Enhanced renal immunocytochemical expression of ANG-(1–7) and ACE2 during pregnancy. *Hypertension.* (2003) 42:749–53. doi: 10.1161/01.HYP.0000085220.53285.11
63. Bhopal R. Covid-19 worldwide: we need precise data by age group and sex urgently. *BMJ.* (2020) 369:m1366. doi: 10.1136/bmj.m1366
64. Moreno R, Alonso JJ, Caballero R, del Corral E, Elízaga J, Asenjo RM, et al. Influence of age and gender on arrival of patients with ST-segment elevation acute myocardial infarction to tertiary centers during COVID-19 pandemic. Experience of Madrid, Spain, STEMI network (Codigo Infarto Madrid). *Am J Emerg Med.* (2020). doi: 10.1016/j.ajem.2020.06.013. [Epub ahead of print].
65. Scully EP, Haverfield J, Ursin RL, Tannenbaum C, Klein SL. Considering how biological sex impacts immune responses and COVID-19 outcomes. *Nat Rev Immunol.* (2020) 20:442–7. doi: 10.1038/s41577-020-0348-8

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Public Health

ORIGINAL RESEARCH
published: 28 July 2020
doi: 10.3389/fpubh.2020.00416

Misconceptions on COVID-19 Risk Among Ugandan Men: Results From a Rapid Exploratory Survey, April 2020

Keneth Iceland Kasozi[1,2], Ewan MacLeod[1], Fred Ssempijja[2], Michael W. Mahero[3], Kevin Matama[4], Grace Henry Musoke[5], Kevin Bardosh[6], Robinson Ssebuufu[7], Florence Wakoko-Studstil[8], Isaac Echoru[9], Emmanuel Tiyo Ayikobua[10], Regan Mujinya[2], Grace Nambuya[2], Hope Onohuean[4], Gerald Zirintunda[11], Justine Ekou[11] and Susan Christina Welburn[1,12]**

[1] Infection Medicine, Deanery of Biomedical Sciences, College of Medicine and Veterinary Medicine, The University of Edinburgh, Edinburgh, United Kingdom, [2] Faculty of Biomedical Sciences, Kampala International University Western Campus, Bushenyi, Uganda, [3] Department of Veterinary Population Medicine, College of Veterinary Medicine, University of Minnesota, St. Paul, MN, United States, [4] School of Pharmacy, Kampala International University Western Campus, Kampala, Uganda, [5] Faculty of Science and Technology, Cavendish University, Kampala, Uganda, [6] Center for One Health Research, School of Public Health, University of Washington, Seattle, WA, United States, [7] Faculty of Clinical Medicine and Dentistry, Kampala International University Teaching Hospital, Bushenyi, Uganda, [8] Department of Criminal Justice & Sociology, College of Letters and Sciences, Columbus State University, Columbus, GA, United States, [9] Department of Anatomy, School of Medicine, Kabale University, Kabale, Uganda, [10] Department of Physiology, School of Health Sciences, Soroti University, Soroti, Uganda, [11] Faculty of Agriculture and Animal Sciences, Busitema University, Tororo, Uganda, [12] Zhejiang University-University of Edinburgh Institute, Zhejiang University School of Medicine, International Campus, Zhejiang University, Haining, China

OPEN ACCESS

Edited by:
Zisis Kozlakidis, International Agency for Research on Cancer (IARC), France

Reviewed by:
Joseph Ntayi, Makerere University, Uganda
David Kitara Lagoro, Gulu University, Uganda

***Correspondence:**
Keneth Iceland Kasozi
kicelandy@gmail.com
Susan Christina Welburn
sue.welburn@ed.ac.uk

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Public Health

Received: *04 June 2020*
Accepted: *13 July 2020*
Published: *28 July 2020*

Citation:
Kasozi KI, MacLeod E, Ssempijja F, Mahero MW, Matama K, Musoke GH, Bardosh K, Ssebuufu R, Wakoko-Studstil F, Echoru I, Ayikobua ET, Mujinya R, Nambuya G, Onohuean H, Zirintunda G, Ekou J and Welburn SC (2020) Misconceptions on COVID-19 Risk Among Ugandan Men: Results From a Rapid Exploratory Survey, April 2020. Front. Public Health 8:416. doi: 10.3389/fpubh.2020.00416

Background: Transmission of COVID-19 in developing countries is expected to surpass that in developed countries; however, information on community perceptions of this new disease is scarce. The aim of the study was to identify possible misconceptions among males and females toward COVID-19 in Uganda using a rapid online survey distributed *via* social media.

Methods: A cross-sectional survey carried out in early April 2020 was conducted with 161 Ugandans, who purposively participated in the online questionnaire that assessed understandings of COVID-19 risk and infection. Sixty-four percent of respondents were male and 36% were female.

Results: We found significant divergences of opinion on gendered susceptibility to COVID-19. Most female respondents considered infection risk, symptoms, severe signs, and death to be equally distributed between genders. In contrast, male respondents believed they were more at risk of infection, severe symptoms, severe signs, and death (52.7 vs. 30.6%, RR = 1.79, 95% CI: 1.14–2.8). Most women did not share this perception and disagreed that males were at higher risk of infection (by a factor of three), symptoms (79% disagree), severe signs (71%, disagree), and death (70.2% disagree). Overall, most respondents considered children less vulnerable (OR = 1.12, 95% CI: 0.55–2.2) to COVID-19 than adults, that children present with less symptoms (OR = 1.57, 95% CI: 0.77–3.19), and that there would be less mortality in children (OR = 0.92, 95% CI: 0.41–1.88). Of female respondents, 76.4% considered mortality from COVID-19 to be different between the young and the elderly (RR = 1.7, 95% CI: 1.01–2.92) and 92.7%

believed young adults would show fewer signs than the elderly, and 71.4% agreed that elderly COVID-19 patients would show more severe signs than the young (OR = 2.2, 95% CI: 1.4, 4.8). While respondents considered that all races were susceptible to the signs and symptoms of infection as well as death from COVID-19, they considered mortality would be highest among white people from Europe and the USA. Some respondents (mostly male 33/102, 32.4%) considered COVID-19 to be a "disease of whites" (30.2%).

Conclusion: The WHO has identified women and children in rural communities as vulnerable persons who should be given more attention in the COVID-19 national response programs across Africa; however, our study has found that men in Uganda perceive themselves to be at greater risk and that these contradictory perceptions (including the association of COVID-19 with "the white" race) suggest an important discrepancy in the communication of *who* is most vulnerable and *why*. Further research is urgently needed to validate and expand the results of this small exploratory study.

Keywords: COVID-19, COVID-19 response in Africa, impact of COVID-19 in Uganda, myths about COVID-19, United Nations emergency appeal response to COVID-19, gender matters in COVID-19 response, impact of COVID-19 in children, efforts to mitigate and adapt to COVID-19

INTRODUCTION

The new pandemic caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged in late 2019, disrupting health systems and critical care, even within the most developed heath systems and economies (1). As of April 22nd, 2020, 2.5 million confirmed cases of COVID-19 have been reported globally (2). Sustained community transmission is expected in low- and middle- income countries (LMICs) where COVID-19 containment strategies continue to be a challenge (3). Extreme mitigation strategies have been put in place in many countries to control COVID-19, to reduce disease transmission and to avoid overburdening healthcare systems including mass lockdowns, curfews, and social distancing measures (4). SARS-CoV-2 and interventions to reduce transmission are negatively impacting already impoverished communities in LMICs and will test heath systems that have little capacity for the management of high dependency patients, or sufficient PPE to protect health workers (5). Interventions will have long-lasting detrimental impacts on LMIC economies, and, in the absence of reliable and efficient tools for early detection of infected and exposed individuals, are likely to extend beyond 2020/21 including in Africa (6).

Africa is vulnerable to being overwhelmed by COVID-19. The World Health Organization (WHO) Director General Dr. Tedros Ghebreyesus, stated that the greatest concern was COVID-19 transmission in countries with weaker health systems than in developed nations (7). On Apr 17, 2020, the WHO estimated 10 million cases of COVID-19 spreading rapidly across Africa and up to 3 million deaths within 6 months (8). Cases are expected to rise quickly due to a chronic lack of testing, lack of personal protective equipment (PPE), and poor patient care facilities of basic equipment to contain the pandemic, such as PPE (9, 10). The ability to contain COVID-19 will depend on the success of social distancing and the ability to diagnose, isolate, and treat cases (11).

Case finding and reporting for COVID-19 in Africa is making less than ideal progress. Data from the African Centers for Disease Control (CDC) shows that while risk of importation of COVID-19 to Africa was lower than that to Europe (1 vs. 11%), response and reaction capacity are also lower; the latter being intrinsically linked to individual country wealth and resources for detection, prevention, and control (12). In late March, Africa had reported 41 local transmissions and only 9 imported cases, by 7 April 2020, 9,888 of 9,971 cases (99.2%) were community acquired with only 83/9,971 cases being imported. As of 18 April 2020, Africa had reported 1,000 deaths with COVID-19 and more than 19,800 cases in 52 out of 54 countries on the African continent (13). With travel restrictions in place, all cases of COVID-19 are considered community acquired (14). While many African nations have employed lessons learned from Ebola (15). COVID-19 is far more challenging to manage. Quantifying the pandemic growth across the African sub-continent and assessing the impact of interventions put in place will be compromised by the lack of diagnostic capacity (16).

Across East Africa, in April 2020, countries lack a coordinated response against COVID-19. While the WHO/AFRO are making strong recommendations, many governments are taking their own approach. The president of Tanzania encouraged people to "pray for 3 days" against COVID-19 and has not imposed any movement restrictions—places of worship remain open (17). In Kenya, only a partial lockdown is in place in major cities and many are not prepared for a total lockdown of the country (18, 19). In Uganda and Rwanda more extreme actions have been taken with total national lockdowns that have involved closure of all non-essential businesses, public transport, and the closure of schools and universities. Only local food stores,

Abbreviations: nCTF, National COVID-19 Task Force; WHO, World Health Organization; LMIC, Low Middle income Countries; BAME, Black, Asian, Minority Ethnic; CDC, Center for Disease Control.

supermarkets, medical, and veterinary supplies are exempt and for these services a mandatory curfew has been imposed. No motor vehicles/motorists are permitted to use public roads unless they are listed among essential service providers authorized by the Office of the President (20–22). Countries not abiding with the social distancing guidelines as recommended by the WHO are putting neighboring countries at risk and compromising health security within the East African Community (EAC). The EAC continues to show a disorganized response against COVID-19 and this high level of disorganization has created confusion in the general public as to what is "true" and "false" COVID-19 information. Rumors and misinformation have spread widely within the community and the media; for example, a religious leader in Uganda claimed that there was no COVID-19 in Uganda and stated that it was just "simple flu" and an individual at Kampala City Council Football Club falsely claimed that Uganda had lost a patient to COVID-19; these culprits are currently in jail awaiting trial for spreading false information (23, 24). Risks and assumptions are causing disharmony in communities, fearful of risk of infection and of the economic consequences of government interventions.

To date, COVID-19 cases and deaths have been greatest in Europe, the Americas, the Western Pacific, the Eastern Mediterranean, South East Asia, and Africa, but the situation is fluid and will change as COVID-19 impacts new regions (25). While most COVID-19 patients have been of European, Asian, and African descent, data from the USA indicates that while African-Americans are at equal risk of infection with SARS-CoV-2, they are at higher risk of severe COVID-19 complications and death. In Illinois, USA, African Americans accounted for 29% of confirmed cases and 41% of deaths, yet comprise 15% of the state's population (26). Similar trends have been observed in Michigan and Wisconsin, USA (27). This is unlikely to have a basis in racial susceptibility but is more likely due to a vulnerability to infection and lack of access to quality medical care—it's not about race but about racism and poverty (28). These observations are of concern for developing countries in Africa and Latin America (29).

The prognosis for COVID-19 infection in Asia and Europe appears to be influenced by sex (being male), pre-existing health conditions (diabetes, cancers, and cardiovascular disease), and age (average age 81 years) (30); other risk factors may include air pollution and smoking (31). Reports released by the Chinese Center for Disease Control and Prevention show that men are more at risk than women (32). Sex differences in males and females in China are supported by the relatively higher antibodies titer generated in females against COVID-19 (33). Reports from Italy showed no significant differences between males and females infected with COVID-19 (34). Older males continue to be disproportionally affected by COVID-19 (35).

In young people (<18 years), reported COVID-19 infections, hospitalization, and death are low (36). COVID-19 generally presents with milder symptoms in children than in adults (3, 7), but the evidence-base is unclear. This does not mean children are immune, and children are considered important asymptomatic carriers able to facilitate SARS-CoV-2 transmission within households (37).

While most COVID-19 infections are mild (with <20% of cases being severe to critical) (38), communities of heavy smokers or those with lung function impairments are believed to be particularly susceptible to complications (39). The WHO has stated that women in Africa are most likely to die from COVID-19 due to sex inequity, chronic poverty among women, weak economic capacity, sexual, and gender-based violence (40). The elderly are more vulnerable to coronavirus (41) and underlying non-communicable diseases that are pervasive in Africa will predispose individuals to complications from SARS-CoV-2 infection.

This study aimed to identify perceptions of COVID-19 risk among Ugandans in order to identify novel strategies to guide the national COVID-19 Task Force (nCTF) to improve, control, and prevent COVID-19 infections.

METHODS

This was a cross sectional study conducted with 161 Ugandan respondents in the second week of April 2020. During this period, COVID-19 infections started to increase in East Africa and Uganda was placed in total lockdown (March–May 2020). A pre-tested online questionnaire using Q-survey® (https://www.qsurvey.qa/home/en) was administered with study participants through online resources i.e., email, Facebook, Twitter, WhatsApp, and Viber. Only Ugandans were included in the study while international residents were excluded from the study, using phone IP addresses, which were automatically generated by the Q survey®. Study participants were encouraged to share the link to the questionnaire with family members and friends to enhance data collection using the same social media platforms. Financial challenges in this period implied that the response rate was low since a majority of Ugandans use prepaid mobile internet connection. The questionnaire was designed using major trending informal statements on COVID-19 to assess the knowledge and perceptions of COVID-19 (**Supplementary File 1**). Completing the questionnaire in full was not a mandatory requirement and some questions could be skipped. Metadata was collected and only study participants whose current location was in Uganda was preserved for statistical analysis.

Statistical Analysis

Data was exported from Q survey in MS Excel and univariate statistics were conducted using WinPEPI® and significance was reported at a 95% confidence interval using questions which were asked to both males and females in Uganda.

RESULTS

COVID-19 Perceptions Amongst Male and Female Ugandans

Of 161 Ugandan respondents, 64% ($n = 103$, 95% CI: 56.3–71.1) were male and 36% were female ($n = 58$, 95% CI: 28.9–43.7). Most were between the ages of 18–30 years (70.5%, 110/161) (**Table 1**). A total of 52.7% of male respondents (54/102) considered males to be more vulnerable to infection with

TABLE 1 | Description of the study participants in Uganda.

Variables		Statistic	Percentage	95% CI
Sex	Male	103[a]	64.0	56.3–71.1
	Female	58[a]	36.0	28.9–43.7
Total		161[a]	100	98.2–100
Age	18–30	110[a]	70.5	60.8–75.2
	31–55	46[a]	28.5	22.0–35.9
	Undeclared	05[a]	3.1	1.2–6.8
Total		161[a]	100	98.2–100
Descriptive of age (years)				
	Mean	28.2[b]		27.1–29.2
	Median	27.0[b]		25.0–29.0
	Minimum	18.0[b]		
	Maximum	55.0[b]		
	25th Percentile	23.0[b]		
	75th Percentile	31.8[b]		
	SEM	0.6[b]		

[a]*Frequency;* [b]*counts. 95% CI, 95% confidence intervals.*

COVID-19 while 70.4% of female respondents (38/54) did not consider males to be more vulnerable; a significant difference between the groups (RR = 1.79, 95% CI: 1.14–2.8). Most female respondents considered infection risk to be equally distributed between genders and there was no significant difference in this perception between male and female respondents.

More male (52.5%, 52/99, 95% CI: 42.7–62.2) respondents believed they were more likely to die from COVID-19 than females (29.6%, 16/54, 95% CI: 18.6–42.8); however 70.2% (40/57) of females disagreed with them on who is more likely to die. Females were half as convinced that men were more likely to die than the male respondents (RR = 1.76, 95% CI: 1.1–2.7). There was no significant difference between the groups of male and female respondents on whether both sexes were equally at risk of death (RR = 0.8, 95% CI: 0.7–1.0). The majority of males (41.4%, 41/99) believed that males present with more severe signs; however, females (71.4%, 40/56) were over two times more likely to disagree with this statement. Female respondents (79%, 45/57) disagreed with the statement that symptoms presented more in males (OR = 1.8, 95% CI: 0.8–4.2). Most female respondents agreed that both sexes showed equal signs for COVID-19 (46/56) however no significant differences were found in males and females (RR = 0.9, 95% CI: 0.8–1.1). The majority of female respondents (n = 40/56, 71.4%, 95% CI: 58.6–82.1) did not think males present with more severe signs of COVID-19 than women, although no significant differences were found (RR = 1.5, 95% CI: 0.9–2.3). Most study participants indicated that both sexes shared the same symptoms (**Table 2**).

Perceptions on COVID-19 Toward Children and Young Adults Amongst Ugandans

Overall, most respondents, 53.5%, 95% CI: 45.7–61.2 (84/157) considered children to be less vulnerable than adults; no significant differences were found between males and females (RR = 1.1, 95% CI: 0.8–1.5). Similar observations were found on participant responses for children being as equally vulnerable as adults. Most respondents considered mortalities to be less in children (male = 63.7%, 65/102, females = 55.4%, 31/56); however, no significant differences were found among those who disagreed with this perception (RR = 1.15, 95% CI: 0.9, 1.5). In addition, most respondents considered children to present with fewer signs of COVID-19 (82/158), and that the signs of infection are not the same in children (77/156), being less severe than in adults (80/155); there were no significant differences between males and females in these responses (**Table 3**).

Perceptions on COVID-19 Risks Among the Elderly

A significantly large proportion of female respondents (76.4%, 42/55) (compared to male respondents 59.4%, 60/101) did not believe mortality rates to be equal in the young and elderly (RR = 1.7, 95% CI: 1.0–2.9). Amongst those who believed that young adults show fewer signs than the elderly, a majority (92.7%, 51/55) of female respondents believed this to be true compared to 84.2% (85/101) of males. Furthermore, a majority of female respondents (71.4%, 40/56)—compared to only 53.1% (52/98) of male respondents agreed that elderly COVID-19 patients would show more severe signs than the young (OR = 2.2, 95% CI: 1.4, 4.8) as shown in **Table 4**. Among those who believed that young adults are more likely to die of COVID-19 than the elderly (19/156), 16% were males while 5.4% were females and there was no significant difference between them.

Perceptions of Risk and Race Among Ugandans

A majority of participants agreed that all races were at risk of COVID-19, however some participants thought that other races were more at risk than others. Amongst those who thought that COVID was a disease of "the whites," a majority of these were males (32.4%, 33/102, 95% CI: 23.82–41.88) compared to 26.3% females (n = 15/57, 95% CI: 16.1–38.9). Furthermore, no significant differences were found between males (5.9%, 6/102) and females (5.3%, 3/57) toward agreement that the disease also affects Blacks. Furthermore, a majority of respondents stated that all races show severe signs of COVID-19 and of these, a majority were females (57.9, 33/57%,) compared to 49.5% (50/101) who were males (**Table 5**).

DISCUSSION

In this study, a majority of study participants were young adults (64%, 103/161) and this was in agreement with statistical reports which have stated that a majority (51.3%) of Ugandans are in the age range of 15–64 years (42). The Uganda National Bureau of Statistics has classified age groups of Ugandans of adults using the 18–30 and 31–64 age groupings for adults (43), demonstrating the importance of young adults in epidemiological surveys. The study showed that a large proportion of males felt they were most vulnerable to COVID-19; however, these sentiments were not shared by women. Findings in the study demonstrate some disparities in COVID-19 risk perceptions at a time when

TABLE 2 | Frequency and percentage of respondents and their risk estimates on signs and symptoms of COVID-19 amongst male and female Ugandans.

Parameter compared to females	Variables	Frequency (%) of study participation response			Risk estimates (95% CI)		
		Male	Female	Total	aR	RR	OR
Age (years)	18–30	65 (65.0)	45 (80.4)	110 (70.5)	−15.4 (−30.7,0.0)	0.9 (0.7–1.0)	0.5 (0.2–1.0)
	31–55	35 (35.0)	11 (19.6)	46 (29.5)			
	Total	100 (100)	56 (100)	156 (100)			
Males more vulnerable	Yes	54 (52.9)	16 (29.6)	70 (44.9)	23.3 (6.3–40.3)	1.8 (1.1–2.8)	2.7 (1.3–5.5)
	No	48 (47.1)	38 (70.4)	86 (55.1)			
	Total	102 (100)	54 (100)	156 (100)			
Equal risk to infection	Yes	68 (67.3)	44 (77.2)	112 (70.9)	−9.9 (−25.5–5.7)	0.9 (0.7–1.1)	0.6 (0.3–1.3)
	No	33 (32.7)	13 (22.8)	46 (29.1)			
	Total	101 (100)	57 (100)	158 (100)			
Males more likely to die	Yes	52 (52.5)	17 (29.8)	87 (55.8)	22.7 (5.9–39.5)	1.8 (1.1–2.7)	2.6 (1.3–5.3)
	No	47 (47.5)	40 (70.2)	69 (44.2)			
	Total	99 (100)	57 (100)	156 (100)			
Both sexes equally die	Yes	61 (59.8)	42 (73.7)	103 (64.8)	−13.9 (−30.1–2.4)	0.8 (0.7–1.0)	0.5 (0.3–1.1)
	No	41 (40.2)	15 (26.3)	56 (35.2)			
	Total	102 (100)	57 (100)	159 (100)			
Males have more signs	Yes	32 (32.3)	12 (21.1)	44 (28.2)	11.3 (−4.1–26.7)	1.5 (0.9–2.7)	1.8 (0.8–4.2)
	No	67 (67.7)	45 (78.9)	112 (71.8)			
	Total	99 (100)	57 (100)	156 (100)			
Males show equal signs	Yes	78 (76.5)	46 (82.1)	124 (78.5)	−5.7 (−20.0–8.7)	0.9 (0.8–1.1)	0.7 (0.3–1.6)
	No	24 (23.5)	10 (17.9)	34 (21.5)			
	Total	102 (100)	56 (100)	158 (100)			
Males with more severe signs	Yes	41 (41.4)	16 (28.6)	57 (36.8)	12.8 (−3.9–29.5)	1.5 (0.9–2.3)	1.8 (0.9–3.6)
	No	58 (58.6)	40 (71.4)	98 (63.2)			
	Total	99 (100)	56 (100)	155 (100)			
Both sexes equal to suffer signs	Yes	69 (68.3)	39 (69.6)	108 (68.8)	−1.3 (−17.8, 15.1)	1.0 (0.8–1.2)	0.9 (0.5–1.9)
	No	32 (31.7)	17 (30.4)	49 (31.2)			
	Total	101 (100)	56 (100)	157 (100)			

Risk estimates conducted with males being the reference category; aR, attributable risk; RR, relative risk; OR, odds ratio; 95% CI, 95% confidence interval.

COVID-19 cases are progressively increasing on the African continent (13). The differences in perception of vulnerability between men and women in our sample is concerning. The WHO has stated that women are at greater vulnerability in Africa (25), a risk perception not shared by our sample of Ugandan respondents (RR = 1.8, 95% CI: 1.14–2.8).

While both males and females recognize the importance of COVID-19 in their households, nearly half (52.9%, 54/102) of male respondents in our study perceived that they were more likely to die from COVID-19 than their female counterparts, while most women disagreed with this perception (RR = 1.8, 95% CI: 1.1–2.7). These findings in Uganda are worrying since a majority of males affected in Europe are elderly (34), and in this exploratory study, this was not the case. Furthermore, a majority of females disagreed with their male counterparts, demonstrating a level of superior knowledge; however, reasons for these disparities were not investigated by the current study. Perceptions of heightened male risk are likely to be influenced by online reports of more males dying than females, in Europe and China (32). It also appears to show an under-appreciation of the structural reasons why women are vulnerable to COVID-19, since they provide the most informal care in families, have limited economic opportunities, and less power in decision making (44, 45).

There is a need to develop novel strategies for communication of COVID-19 risk in Africa (5). The prognosis for COVID-19 patients in Asia and Europe appears to be affected mainly by sex, pre-existing health conditions, such as diabetes, cancers, and cardiovascular disease and being elderly (30), conditions which are also progressively rising in the African continent, despite poor prioritization of health service systems (46). However, women seek more health services than men (47). This may explain why women are able to mount stronger immunity than men (48); however, a young population in Africa is bound to have its own infection dynamics. Recent findings from Italy show no significant differences between genders (34). Symptomology in COVID-19 depends heavily on the immune status of a patient due to risky lifestyles like smoking and air pollution, and not necessarily on gender (33). In East African communities, household air pollution remains a public health threat especially

TABLE 3 | Descriptive statistics and risk estimates on perceptions of COVID-19 in children and adults amongst male and female Ugandans.

Parameter compared to adults	Variables	Frequency (%) of study participation response			Risk estimates (95% CI)		
		Male	Female	Total	aR	RR	OR
Children more vulnerable	Yes	48 (47.5)	25 (44.6)	73 (46.5)	2.9 (−14.8–20.5)	1.1 (0.8–1.5)	1.1 (0.6–2.2)
	No	53 (52.5)	31 (55.4)	84 (53.5)			
	Total	101 (100)	56 (100)	157 (100)			
Children equally vulnerable	Yes	41 (40.2)	20 (36.4)	61 (38.9)	3.8 (−10.9–18.6)	1.1 (0.7–1.7)	1.2 (0.6–2.5)
	No	61 (59.8)	35 (63.6)	96 (61.1)			
	Total	102 (100)	55 (100)	157 (100)			
Children die less	Yes	65 (63.7)	31 (55.4)	96 (60.8)	8.4 (−9.0–25.8)	1.2 (0.9–1.5)	1.4 (0.7–2.9)
	No	37 (36.3)	25 (44.6)	62 (39.2)			
	Total	102 (100)	56 (100)	158 (100)			
Children die equally	Yes	44 (44.4)	26 (46.4)	70 (45.2)	−2.0 (−19.7–15.7)	1.0 (0.−1.4)	0.9 (0.4–1.9)
	No	55 (55.6)	30 (30.0)	85 (54.8)			
	Total	99 (100)	56 (100)	155 (100)			
Children less signs	Yes	57 (55.9)	25 (44.6)	82 (51.9)	11.2 (−6.3–28.8)	1.3 (0.9–1.8)	1.6 (0.8–3.2)
	No	45 (44.1)	31 (55.4)	76 (48.1)			
	Total	102 (100)	56 (100)	158 (100)			
Children equal signs	Yes	52 (52)	27 (48.2)	79 (50.6)	3.8 (−14.0–21.5)	1.1 (0.8–1.5)	1.2 (0.6–2.4)
	No	48 (48)	29 (51.8)	77 (49.4)			
	Total	100 (100)	56 (100)	156 (100)			
Children less severe signs	Yes	55 (55)	25 (45.5)	80 (51.6)	9.5 (−8.2–27.3)	1.2 (0.9–1.7)	1.5 (0.7–3.0)
	No	45 (45)	30 (54.5)	75 (48.4)			
	Total	100 (100)	55 (100)	155 (100)			
Children equally severe signs	Yes	48 (48)	28 (50)	76 (76)	−2.0 (−19.7–15.7)	1.0 (0.7–1.3)	0.9 (0.5–1.9)
	No	52 (52)	28 (50)	80 (80)			
	Total	100 (100)	56 (100)	156 (100)			

Risk estimates conducted with males being the reference category; aR, attributable risk; RR, relative risk; OR, odds ratio; 95% CI, 95% confidence interval.

in slums and rural communities where the use of firewood and charcoal continues to be routine (49). In Malaysia, knowledge has been shown to affect practice to minimize exposure to air pollutants (50), while a recent study in Uganda on COVID-19 has shown that knowledge affects practices promoted by the WHO against COVID-19 amongst market vendors (51).

Most male and female Ugandans in our sample believed that children below 18 years were less vulnerable to COVID-19 infections and that if they contracted the illness, they would be faced with a milder infection and that less would die from the disease. This is a common global perception that has been communicated by the media and many health agencies around the world in 2020 and is in agreement with studies that have shown a low infection rate in children (36). However, a significant number of women in this survey considered children to be equally vulnerable than adults and that children die more from the disease than adults (RR = 1.2, 95% CI: 0.9, 1.5). This may be due to maternal sentiments, communicated by women and not shared by men in Uganda, where men are often believed to show less empathy toward children (52).

The general opinion that elderly persons are more vulnerable to COVID-19 than young people is in agreement with recent epidemiological findings (42). Elderly individuals are known to be at high risk for COVID-19 (30). In general, women appear more knowledgeable than men on SARS-CoV-2; they may have a greater interest in health-related topics and show more health seeking behavior than men (51). Such misconceptions may have significant and far-reaching influence on health-seeking behavior (53).

It has been proposed that conception is functional and if people can solve problems within their existing conceptual environment, then the drive to change one's opinion becomes weak, although this does not help in solving a current problem (54). Thus, this theory of conceptual change is embedded in a set of epistemological assumptions that are far more generalizable than our application to misconceptions has exploited. These epistemological assumptions suggest that the basic problem of understanding cognitive development is to understand how the components of an individual's conceptual ecology interact and develop and how the conceptual ecology interacts with experience (55).

Of concern in this study is the perception among 30.2% (48/159) of respondents, particularly men, that COVID-19 is a "white-man's disease;" these feelings were strongest amongst males. These sentiments reflect the present disunified response against COVID-19 in East African states. In Tanzania and

TABLE 4 | Perceptions on COVID-19 presentation in young adults and the elderly in Ugandans.

Parameter as compared to the elderly	Variable	Frequency (%) of study participation response			Risk estimates (95% CI)		
		Male	Female	Total	aR	RR	OR
Young adults are more likely to die	Yes	16 (16.0)	3 (5.4)	19 (12.2)	10.6 (0.0–21.3)	3.0 (0.9–9.8)	3.4 (0.9–18.8)
	No	84 (84.0)	53 (94.6)	137 (87.8)			
	Total	100 (100)	56 (100)	156 (100)			
Young adults are equally vulnerable to dying	Yes	41 (40.6)	13 (23.6)	54 (34.6)	17.0 (0.8–33.1)	1.7 (1.0–2.9)	2.2 (1.0–5.0)
	No	60 (59.4)	42 (76.4)	102 (65.4)			
	Total	101 (100)	55 (100)	156 (100)			
Young adults show more signs	Yes	16 (15.8)	4 (7.3)	20 (12.8)	08.6 (−2.7–19.9)	2.2 (0.8–6.2)	2.4 (0.7–10.4)
	No	85 (84.2)	51 (92.7)	136 (87.2)			
	Total	101 (100)	55 (100)	156 (100)			
Young adults equally show signs	Yes	45 (45.5)	17 (31.5)	62 (40.5)	14.0 (−03.3–31.2)	1.4 (0.9–2.3)	1.8 (0.93.9)
	No	54 (54.5)	37 (68.5)	91 (59.5)			
	Total	99 (100)	54 (100)	153 (100)			
Young adults are more vulnerable to severe signs	Yes	19 (19.4)	5 (9.3)	24 (15.8)	10.1 (−02.3–22.6)	2.1 (0.8–5.3)	2.4 (0.8–8.6)
	No	79 (80.6)	49 (90.7)	128 (84.2)			
	Total	98 (100)	54 (100)	152 (100)			
Young adults equally show severe signs	Yes	46 (46.9)	16 (28.6)	62 (40.3)	18.4 (1.5–35.2)	1.6 (1.0–2.6)	2.2 (1.0–4.8)
	No	52 (53.1)	40 (71.4)	92 (59.7)			
	Total	98 (100)	56 (100)	154 (100)			

Risk estimates conducted with males being the reference category; aR, attributable risk; RR, relative risk; OR, odds ratio; 95% CI, 95% confidence interval.

TABLE 5 | Description of participant responses on race amongst male and female Ugandans.

Parameter compared to race	Variable	Frequency (%) of respondents			Risk estimates		
		Male	Female	Total	aR	RR	OR
Vulnerable	White	33 (32.4)	15 (26.3)	48 (30.2)	6.5	1.3	1
	All races	53 (52.0)	33 (57.9)	86 (54.1)	−4.9	0.9	0.7
	Blacks	6 (5.9)	3 (5.3)	9 (5.7)	−1.0	0.9	0.7
	Not Sure	10 (9.8)	6 (10.5)	16 (10.1)	−0.5	0.9	0.8
	Total	102 (100)	57 (100)	159 (100)			
Susceptibility to death	White	37 (36.6)	13 (23.2)	50 (31.8)	13.4	1.6	1
	All races	50 (49.5)	32 (57.1)	82 (52.2)	−7.6	0.9	0.5
	Blacks	5 (5.0)	2 (3.6)	7 (4.5)	1.4	1.4	0.9
	Not Sure	9 (8.9)	9 (16.1)	18 (11.5)	−7.2	0.6	0.4
	Total	101 (100)	56 (100)	157 (100)			
More signs and symptoms	White	34 (33.7)	15 (26.8)	49 (31.2)	6.7	1.3	1
	All races	54 (53.5)	31 (55.4)	85 (54.1)	−1.9	1.0	0.8
	Blacks	6 (5.9)	2 (3.6)	8 (5.1)	2.4	1.7	1.3
	Not Sure	7 (6.9)	8 (14.3)	15 (9.6)	−7.4	0.5	0.4
	Total	101 (100)	56 (100)	157 (100)			
Severe signs	White	38 (37.6)	16 (28.1)	54 (34.2)	1.0	1.3	1
	All races	50 (49.5)	33 (57.9)	83 (52.5)	−8.4	0.9	0.6
	Blacks	6 (5.9)	3 (5.3)	9 (5.7)	0.7	1.1	0.8
	Not Sure	7 (6.9)	5 (8.8)	12 (7.6)	−1.8	0.8	0.6
	Total	101 (100)	57 (100)	158 (100)			

Risk estimates conducted using a 2 x k analysis. aR, attributable risk; RR, relative risk; OR, odds ratio; 95% CI, 95% confidence interval.

Burundi prayers are being promoted for people to seek divine intervention (17). Many believe that Africa will "be spared" COVID-19, since the disease originated in Asia before spreading to Europe (1), and following reports that malaria endemic regions would be protected, from generational exposure to chloroquine and hydroxychloroquine amongst Africans, since these drugs were reported to have some success in COVID-19 treatment (56, 57). That Ugandans perceive COVID-19 and infection of "whites" or the "other" is in direct contradiction of data coming from the USA and UK where Black, Asian, and minority ethnic (BAME) communities have been hit hard with infection.

Projections, from the WHO and others, indicate over 10 million cases of COVID-19 in Africa (8), and 3 million COVID-19 related deaths in the coming months of 2020 (11). Quantifying pandemic growth and assessment of interventions put in place (16) will not be straightforward in low- and middle- income settings faced with challenges of access to testing and reporting of cases and deaths. While many African nations have indeed learned from experiences of Ebola (15), SARS-CoV-2 is a far greater challenge. The virus itself and interventions that have been put in place are both new and difficult to manage long-term in LMICs. Disunity of policies across Sub-Saharan Africa (17, 18, 20, 21), will not enable disease containment necessary for economic and social recovery and will fuel sustained community transmission (3).

Study Limitations

The study was conducted through an Online application, meaning that Ugandans without smart phones connected to the internet were not able to participate. Recruitment was achieved through sharing of the online link *via* social media and email platforms and data in this study was generally from the same age group i.e., 18–31 years. The sample size was small and further large-scale studies are needed to extend this exploratory study. Furthermore, results should be approached with caution until more large-scale studies are conducted which would include asymptomatic variables not investigated in the current study.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

Expediated ethical approval was acquired from the Institutional Review Board of Kampala International University under ID: Nr.UG-REC-023/201914. Consent to participate was acquired through online acceptance to participate in the study.

AUTHOR CONTRIBUTIONS

KK and SW conceptualized the study. KK, SW, and FS designed the study. KK, FS, KM, GM, RS, IE, EA, RM, GN, HO, GZ, and JE conducted data acquisition. KK, EM, KB, MM, and SW conducted statistical analysis. KK, FW-S, EM, KB, MM, and SW conducted interpretation. KK, SW, and KB drafted the initial manuscript. KK, EM, KB, FS, MM, KM, GM, RS, FW-S, IE, EA, RM, GN, HO, GZ, JE, and SW revised the manuscript for intellectual content and approved the final version for publication. All authors agree to be accountable for all aspects of the work.

FUNDING

This work was supported by Zhejiang University Education Foundation Emergency Research Fund (SW and KB); Global Challenges Research Fund and the University of Edinburgh.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2020.00416/full#supplementary-material

REFERENCES

1. Chen N, Zhou M, Dong X, Qu J, Gong F, Han Y, et al. Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study. *Lancet.* (2020) 395:507–13. doi: 10.1016/S0140-6736(20)30211-7
2. Kate M, Siddiqui U, Pietromarchi V. Confirmed coronavirus cases top 2.5m worldwide: live updates. *Aljazeera.* (2020). Available online at: https://www.aljazeera.com/news/2020/04/coronavirus-dead-world-exceeds-170000-live-updates-200421005048334.html (accessed April 24, 2020).
3. Salzberger B, Glück T, Ehrenstein B. Successful containment of COVID-19: the WHO-report on the COVID-19 outbreak in China. *Infect.* (2020) 48:151–3. doi: 10.1007/s15010-020-01409-4
4. Roy MA, Hans H, Don K, Déirdre TH. How will country-based mitigation measures influence the course of the COVID-19 epidemic? *Lancet.* (2020) 395:931–4. doi: 10.1016/S0140-6736(20)30567-5
5. Kasozi KI, Mujinya R, Bogere P, Ekou J, Zirintunda G, Ahimbisibwe S, et al. (2020) Pandemic panic and anxiety in developing countries. Embracing One Health offers practical strategies in management of COVID-19 for Africa. *Pan Afr Med J.* 35(Suppl 2):3. doi: 10.11604/pamj.2020.35.3.22637
6. World Bank Group. "Assessing the economic impact of COVID-19 and policy responses in sub Saharan Africa," in *Africa's Pulse*, Vol. 21. Office of the Chief Economist for the African Region (2020), 62. Available online at: https://reliefweb.int/sites/reliefweb.int/files/resources/9781464815683.pdf (accessed April 10, 2020).
7. Kapata N, Chikwe I, Francine N, Tajudeen R, Pascalina C-K, Mwaba P, et al. Is Africa prepared for tackling the COVID-19 SARS-CoV-2) epidemic. Lessons from past outbreaks, ongoing pan-African public health efforts, and implications for the future. *Int J Infect Dis.* (2020) 93:233–6. doi: 10.1016/j.ijid.2020.02.049
8. Rogan W. Africa coronavirus cases could hit 10 million in six months: WHO. *Aljezera.* (2020). Available online at: https://www.aljazeera.com/news/2020/04/africa-coronavirus-cases-hit-10-million-months-200417055006127.html (accessed April 18, 2020).
9. Graeme W. Think 168,000 ventilators is too few? Try three. *Atlantic.* (2020). Available online at: https://www.theatlantic.com/ideas/archive/2020/04/why-covid-might-hit-african-nations-hardest/609760/ (accessed April 17, 2020).
10. Chris H. *Will Lack of Food Make COVID-19 a Worse Crisis in Africa?* Oxfarm (2020). Available online at: https://www.oxfamamerica.org/explore/stories/will-lack-food-make-covid-19-worse-crisis-africa/ (accessed April 24, 2020).

11. Joe P. Three million people could die from coronavirus in africa unless the spread is contained, UN report warns - as WHO officials say the continent could be the next COVID-19 epicentre. *Daily Mail.* (2020). Available online at: https://www.dailymail.co.uk/sciencetech/article-8229311/UN-report-warns-THREE-MILLION-people-die-coronavirus-Africa.html (accessed April 18, 2020).
12. Gilbert M, Pullano G, Pinotti F, Valdano E, Poletto C, Boëlle PY, et al. Preparedness and vulnerability of African countries against importations of COVID-19: a modelling study. *Lancet.* (2020) 395:871–7. doi: 10.1016/S0140-6736(20)30411-6
13. Bashir A, Umar H. Africa deaths above 1,000, including nigerian chief of staff. *CTV News.* (2020). Available online at: https://www.ctvnews.ca/health/coronavirus/africa-deaths-above-1-000-including-nigerian-chief-of-staff-1.4901998 (accessed April 18, 2020).
14. Africa News. Coronavirus - Africa: update on the ongoing novel coronavirus (COVID-19) global epidemic, brief 13. *Africa News.* (2020). Available online at: https://www.africanews.com/2020/04/14/coronavirus-africa-update-on-the-ongoing-novel-coronavirus-covid-19-global-epidemic-brief-13// (accessed April 24, 2020).
15. Miriam S. *In the Face of Coronavirus, African Countries Apply Lessons From Ebola Response*. World Bank (2020). Available online at: https://www.worldbank.org/en/news/feature/2020/04/03/in-the-face-of-coronavirus-african-countries-apply-lessons-from-ebola-response (accessed April 17, 2020).
16. Kaan D. *ANALYSIS - COVID-19 Pandemic: Great Danger Awaits Africa*. Anadolu Agency (2020). Available online at: https://www.aa.com.tr/en/africa/analysis-covid-19-pandemic-great-danger-awaits-africa/1789840 (accessed April 17, 2020).
17. Beatrice M. *Covid-19: Magufuli Declares Three Days of National Prayers*. East African (2020). Available online at: https://www.theeastafrican.co.ke/news/ea/Covid19-Tanzania-declares-three-days-of-national-prayers/4552908-5526674-7pncj5/index.html (accessed April 17, 2020).
18. Jill N. COVID-19: Kenya's cases rise to 234 as nine more test positive. *East Africa.* (2020). Available online at: https://www.theeastafrican.co.ke/news/ea/Kenya-Covid19-cases-rise-to-234/4552908-5526232-rsdjyfz/index.html (accessed April 17, 2020).
19. Paul W. Kenyans want total lockdown that they're not prepared for. *Daily Nation.* (2020). Available online at: https://allafrica.com/stories/202004060620.html (accessed April 24, 2020).
20. Ivan RM. Rwanda extends lockdown as coronavirus cases jump to 82. *East African.* (2020). Available online at: https://www.theeastafrican.co.ke/news/ea/Rwanda-extends-lockdown-as-coronavirus-cases-jump-to-82/4552908-5511868-mg0i63z/index.html (accessed April 17, 2020).
21. Raymond T. COVID-19: museveni extends Uganda lockdown to may 5. *East African.* (2020). Available online at: https://www.theeastafrican.co.ke/news/ea/Museveni-extends-Uganda-lockdown-to-May-5/4552908-5523958-47ymme/index.html (accessed April 17, 2020).
22. Javira S. BREAKING! Museveni announces 14-day total lockdown in Uganda, suspends private transport, people movements to stem coronavirus spread. *PML Daily.* (2020). Available online at: https://www.pmldaily.com/news/2020/03/breaking-museveni-bans-private-transport-issues-tight-additional-measures.html (accessed April 24, 2020).
23. Keneth K. KCCA payroll officer arrested for spreading fake news on first COVID-19 death in Uganda. *Nile Post.* (2020). Available online at: https://nilepost.co.ug/2020/04/13/kcca-payroll-officer-arrested-for-spreading-fake-news-on-first-covid-19-death-in-uganda/ (accessed April 24, 2020).
24. URN. *Pastor Yiga Arrested for Misleading Public on COVID-19.* (2020). Available online at: https://www.monitor.co.ug/News/National/Pastor-Yiga-arrested-misleading-public-COVID19/688334-5507644-1380ounz/index.html (accessed April 24, 2020).
25. WHO. *Corona Virus Disease 2019 (COVID-19) Situation Report- 88.* World Health Organization (2020). Available online at: https://www.who.int/docs/default-source/coronaviruse/situation-reports/20200417-sitrep-88-covid-191b6cccd94f8b4f219377bff55719a6ed.pdf?sfvrsn=ebe78315_6 (accessed April 7, 2020).
26. Illinois Department of Public Health. *Corona Virus Disease 2019 (COVID-19).* Illinois Department of Public Health (2020). Available online at: http://www.dph.illinois.gov/ (accessed April 16, 2020).
27. Akilah J, Buford T. *Early Data Shows African Americans Have Contracted and Died of Coronavirus at an Alarming Rate*. ProPublica (2020). Available online at: https://www.propublica.org/article/early-data-shows-african-americans-have-contracted-and-died-of-coronavirus-at-an-alarming-rate (accessed April 7, 2020).
28. Clarence G. It's about racism, not race, when coronavirus hits communities of color hard |column. *Tampa Bay Times.* (2020). Available online at: https://www.tampabay.com/opinion/2020/04/15/its-about-racism-not-race-when-coronavirus-hits-communities-of-color-hard-column/ (accessed April 24, 2020).
29. Payne C. COVID-19 in Africa. *Nat Hum Behav.* (2020) 4:436–7. doi: 10.1038/s41562-020-0870-5
30. Annette Y. *Coronavirus: Why Do More Men Die of Covid-19 Than Women?* France24 (2020). Available online at: https://www.france24.com/en/20200331-coronavirus-why-do-more-men-die-of-covid-19-than-women (accessed April 17, 2020).
31. Jeffrey N. *Air Pollution and Smoking May Increase Coronavirus Risks, Worsen Outcomes.* University of California, San Francisco (2020). Available online at: https://www.ucsf.edu/news/2020/04/417106/air-pollution-and-smoking-may-increase-coronavirus-risks-worsen-outcomes (accessed April 24, 2020).
32. Carmen N. Do women and men have a coronavirus risk gap? *Forbes.* (2020). Available online at: https://www.forbes.com/sites/carmenniethammer/2020/03/06/do-women-and-men-have-a-coronavirus-risk-gap/#21013e7f6826 (accessed April 17, 2020).
33. Zeng F, Dai C, Cai P, Wang J, Xu L, Li J, et al. A comparison study of SARS-CoV-2 IgG antibody between male and female COVID-19 patients: a possible reason underlying different outcome between sex. *J Med Virol*. (2020). doi: 10.1002/jmv.25989
34. Statista Research Department. *Distribution of Coronavirus Cases in Italy as of April 15, 2020, by Gender*. Statista Research Department (2020). Available online at: https://www.statista.com/statistics/1103031/coronavirus-cases-distribution-by-gender-italy/ (accessed April 17, 2020).
35. WHO African Region. External situation Report 5. In: *COVID-19 Situation Update.* (2020). Available online at: https://www.afro.who.int/health-topics/coronavirus-covid-19 (accessed April 17, 2020).
36. Sharon B. *Who Is Getting Sick, and How Sick? A Breakdown of Coronavirus Risk by Demographic Factors*. STAT Reports (2020). Available online at: https://www.statnews.com/2020/03/03/who-is-getting-sick-and-how-sick-a-breakdown-of-coronavirus-risk-by-demographic-factors/ (accessed April 17, 2020).
37. Kelvin AA, Halperin S. COVID-19 in children: the link in the transmission chain. *Lancet Infect Dis*. (2020) 20:633–4. doi: 10.1016/S1473-3099(20)30236-X
38. BBC. Coronavirus: largest study suggests elderly and sick are most at risk. *BBC News*. (2020). Available online at: https://www.bbc.com/news/world-asia-china-51540981 (accessed April 16, 2020).
39. Anjana A. Coronavirus business update. Financial Times (2020). Available online at: https://www.ft.com/content/a32e62b4-4be9-11ea-95a0-43d18ec715f5 (accessed April 16, 2020).
40. WHO. *Corona Virus Disease 2019 (COVID-19) Situation Report- 88*. World Health Organization (2020). Available online at: https://www.who.int/docs/default-source/coronaviruse/situation-reports/20200417-sitrep-88-covid-191b6cccd94f8b4f219377bff55719a6ed.pdf?sfvrsn=ebe78315_6 (accessed Tuesday April 7, 2020).
41. Kate W. An expert explains: how to help older people through the COVID-19 pandemic. *World Economic Forum.* (2020). Available online at:https://www.weforum.org/agenda/2020/03/coronavirus-covid-19-elderly-older-people-health-risk/ (accessed April 17, 2020).
42. Uganda Ministry of Health. *Uganda Population-Based HIV Impact Assessement (UPHIA) 2016-2017*. United States Department of Health and Human Services (2019). Available online at: https://phia.icap.columbia.edu/wp-content/uploads/2019/07/UPHIA_Final_Report_Revise_07.11.2019_Final_for-web.pdf (accessed April 13, 2020).
43. Uganda Bureau of Statistics. *Good Governance; A Prerequisite to Harness the Demographic Dividend for Sustainable Development*. State of Uganda Population Report 2018 (2018). Available online at: http://npcsec.go.ug/wp-content/uploads/2013/06/SUPRE-2018-.pdf (accessed April 14, 2020).

44. United Nations. *The Impact of COVID-19 on Women*. DESA Policy Breif (2020). Available online at: https://www.un.org/sexualviolenceinconflict/wp-content/uploads/2020/06/report/policy-brief-the-impact-of-covid-19-on-women/policy-brief-the-impact-of-covid-19-on-women-en-1.pdf (accessed April 13, 2020).
45. Lewin SR, Rasmussen TA. COVID-19 : the gendered impacts of the outbreak. *Lancet.* (2020) 395:846–8. doi: 10.1016/S0140-6736(20)30526-2
46. Neo T. *We Need to Fix a Patient Data Gap to Cope With the Rise of "Rich" Diseases in Africa.* Quartz Africa (2020). Available online at: https://qz.com/africa/989475/cancer-diabetes-and-heart-diseases-rise-in-africa-needs-better-patient-data-to-cope/ (accessed April 24, 2020).
47. Galdas PM, Marshall P. Men and health help-seeking behaviour: literature review. *J Adv Nurs.* (2005) 46:616–23. doi: 10.1111/j.1365-2648.2004.03331.x
48. Roni CR. *Why the Coronavirus Seems to Hit Men Harder Than Women*. New York Times (2020). Available online at: https://www.nytimes.com/2020/02/20/health/coronavirus-men-women.html (accessed April 17, 2020).
49. Muindi K, Kimani-murage E, Egondi T, Rocklov J, Nawi N. Household air pollution: sources and exposure levels to fine particulate matter in nairobi slums. *Toxics.* (2016) 4:12–4. doi: 10.3390/toxics4030012
50. De Pretto L, Acreman S, Ashfold MJ, Mohankumar SK, Campos-Arceiz A. The link between knowledge, attitudes and practices in relation to atmospheric haze pollution in Peninsular Malaysia. *PLoS ONE.* (2015) 10:1–18. doi: 10.1371/journal.pone.0143655
51. Usman IM, Ssempijja F, Ssebuufu R, Lemuel AM, Archibong VB, Ayikobua ET, et al. Community drivers affecting adherence to WHO guidelines against COVID-19 amongst rural Ugandan Market Vendors. *Front Public Health.* (2020) 8:13. doi: 10.3389/fpubh.2020.00340
52. Mestre MV, Samper P, Frías MD, Tur AM. Are women more empathetic than men? A longitudinal study in adolescence. *Spanish J Psychol.* (2009) 12:76–83. doi: 10.1017/S1138741600001499
53. Özdemir G, Clark DB. An overview of conceptual change theories. *Eurasia J Math Sci Technol Educ.* (2007) 3:351–61. doi: 10.12973/ejmste/75414
54. Hewson PW. A conceptual change approach to learning science. *Eur J Sci Educ.* (1981) 3:383–96. doi: 10.1080/0140528810304004
55. Strike KA, Posner GJ. A revisionist theory of conceptual change. In: Duschl R, Hamilton R, editors, *Philosophy of Science, Cognitive Psychology, and Educational Theory and Practice.* Albany, NY: State University of New York Press (1992). p. 147–76.
56. WHO. *Malaria and the COVID-19 Pandemic*. WHO (2020). Available online at: https://www.who.int/news-room/q-a-detail/malaria-and-the-covid-19-pandemic (accessed April 24, 2020).
57. Ludvigsson JF. Systematic review of COVID-19 in children shows milder cases and a better prognosis than adults. *Acta Paediatr Int J Paediatrics.* (2020) 109:1088–95. doi: 10.1111/apa.15270

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Medicine

OPINION
published: 28 July 2020
doi: 10.3389/fmed.2020.00453

Spironolactone: An Anti-androgenic and Anti-hypertensive Drug That May Provide Protection Against the Novel Coronavirus (SARS-CoV-2) Induced Acute Respiratory Distress Syndrome (ARDS) in COVID-19

Flavio A. Cadegiani[1,2]*, *Carlos G. Wambier*[3] *and Andy Goren*[3,4]

[1] *Department of Endocrinology, Federal University of São Paulo, São Paulo, Brazil,* [2] *Corpometria Institute, Brasília, Brazil,* [3] *Department of Dermatology, Warren Alpert Medical School of Brown University, Providence, RI, United States,* [4] *Applied Biology Inc., Irvine, CA, United States*

Keywords: COVID-19, SARS-CoV-2, coronavirus, pandemic, spironolactone, ARDS (acute respiratory distress syndrome), androgen receptor antagonist, TMPRSS2

OPEN ACCESS

Edited by:
Ata Murat Kaynar, University of Pittsburgh School of Medicine, United States

Reviewed by:
Torsten Gerriet Blum, Helios Kliniken, Germany

***Correspondence:**
Flavio A. Cadegiani
flavio.cadegiani@unifesp.br;
f.cadegiani@gmail.com

Specialty section:
This article was submitted to Pulmonary Medicine, a section of the journal Frontiers in Medicine

Received: *20 April 2020*
Accepted: *08 July 2020*
Published: *28 July 2020*

Citation:
Cadegiani FA, Wambier CG and Goren A (2020) Spironolactone: An Anti-androgenic and Anti-hypertensive Drug That May Provide Protection Against the Novel Coronavirus (SARS-CoV-2) Induced Acute Respiratory Distress Syndrome (ARDS) in COVID-19. Front. Med. 7:453. doi: 10.3389/fmed.2020.00453

INTRODUCTION

At the onset of the COVID-19 pandemic, mortality following infection of severe acute respiratory coronavirus (SARS-CoV-2) was thought to be solely associated with aging and pre-existing conditions; however, as the pandemic ensued, several large scale epidemiological observations eluded to additional atypical risk factors, particularly hypertension, obesity, and male gender (1–11).

SARS-CoV-2: CURRENT KNOWLEDGE ON THE MECHANISMS OF ACTION

The peculiarities and complexity of SARS-CoV-2 infection patterns precluded definitive findings regarding the mechanisms of infectivity. Current literature suggests that angiotensin-converting enzyme-2 (ACE2) receptor and transmembrane serine protease 2 (TMPRSS2) are the key for SARS-CoV-2 cell entry (12–19). While ACE2 is the coupling site of the spike protein of SARS-CoV-2, TMPRSS2 facilitates SARS-CoV-2 spikes and ACE2 for viral cell entry. Although ACE2 expression is present diffusely, up to 80% of its expression is located in the type-2 pneumocytes (12, 17), which may explain why COVID-19 is predominantly pulmonary, although SARS-COV-2 may affect any organ and system. TMPRSS2 activity is modulated by androgens, which may justify why males are overrepresented among severe COVID-19 infected patients (20).

Current understanding of SARS-CoV-2 allows the division of COVID-19 into two phases (12–18). In a first, early phase, which corresponds to the period of SARS-CoV-2 cell entry, lung membrane-attached ACE2 expression seems to be positively correlated with virus infectivity, while the balance between circulating ACE2, that could protect from lung infectivity by coupling with SARS-CoV-2 and precluding from the entry into the pneumocytes (13–16), and membrane-attached ACE2, may also be relevant.

In a second phase, represented by the inflammatory and immunological responses to SARS-CoV-2 infection, ACE2 is downregulated due to the entry into cell cytoplasm when coupled with the virus. In opposition to the first phase, in the second phase, lung-attached ACE2 expression may be positively correlated with better clinical outcomes, since ACE2 may limit the cytokine storm that underlies the Acute Respiratory Distress Syndrome (ARDS) in COVID-19, while the

balance between proinflammatory angiotensin II–angiotensin receptor type 1 (AT1) axis, and the anti-inflammatory angiotensin 1–7—G-coupled Mas receptor (angiotensin 1–7 receptor) axis may also be crucial for level of severity of the second phase (13, 15–19).

SARS-CoV-2: THE LINK BETWEEN MECHANISMS OF ACTION AND RISK FACTORS

The Renin-Angiotensin-Aldosterone System (RAAS) has been shown to be central in COVID-19, since three of the key modulators of SARS-CoV-2 infectivity–angiotensin 1–7, ACE2, and AT1—belong to the RAAS, in addition to the TMPRSS2 expression (12–19).

Disruption of RAAS and ACE2 expression abnormalities are likely the underlying mechanism that links hypertension and obesity as important risk factors for COVID-19 (21–29). Conversely, TMPRSS2 overexpression in response to exposure to androgens may justify the higher occurrence of COVID-19 complications in males (30–33), which can be reinforced by the fact that males under androgen deprivation therapies such as for prostate cancer may experiment decreased risk for ARDS when compared to age-, sex-, and comorbidities-matched subjects (33).

A pro-thrombotic state, and endothelial, hematological, kidney, hepatic, cardiovascular, gonadal, neurological, and gastrointestinal manifestations in COVID-19 are at least partially mediated by ACE2 and TMPRSS2 expressions (34–60).

In summary, aberrancies in ACE2 expression, unbalance between angiotensin II and angiotensin 1–7 levels, and overexpression of TMPRS22 seem to be key factors for the severity of clinical manifestations in COVID-19.

SPIRONOLACTONE AS A CANDIDATE AGAINST COVID-19

Drugs that address ACE2, any sight of the RAAS, or TMPRSS2 expression are potential candidates for COVID-19. In this context, the use of old drugs against COVID-19 may present major potential benefits over novel drugs for some reasons, including: (1) The well-established long-term safety profile (2) Extensively described risks and contraindications, which allows to prevent its use when contraindicated and monitor for risks directedly; and (3) The lower cost of old, non-patented drugs allows its massive use in public health systems, when clinically indicated.

These observations combined with our understanding of SARS-CoV-2 molecular mechanism of infectivity lead us to believe that spironolactone is an ideal candidate drug for the prophylactic treatment of SARS-CoV-2.

Spironolactone is a safe and well-tolerated anti-hypertensive and anti-androgenic drug used since 1959, that is effective to maintain normal blood levels (61–63), address heart function, and provide cardio- and renoprotection (64–68).

While spironolactone is a safe and unexpensive option, it may act in multiple sites against COVID-19, including: (1) Favorable patterns of ACE2 expression, including potential increase of circulating ACE2, enhancing its potential protective role in SARS-CoV-2, once plasma ACE2 may couple to SARS-CoV-2 and avoid its entry in the cells (24, 69–74); (2) Downregulation of the androgen-mediated TMPRSS2 due to its antiandrogenic activity (75–77), without the adverse events of male sexual castration; (3) Mitigation of the deleterious effects of obesity on the RAAS, possibly reducing obesity-related COVID-19 complications (78, 79); (4) Direct anti-inflammatory and anti-viral effects that could directly avoid pulmonary complications of COVID-19 (80–90).

Hence, spironolactone meets corresponding epidemiological data, mechanistical plausibility, and sufficient safety profile to become a candidate against COVID-19.

For the proper management of spironolactone during COVID-19, since spironolactone mostly targets the virus entry in the cells, which is the hallmark of the first phase of Covid-19, spironolactone should be preferably started during the earlier stages of the infection, prior to the complications of respiratory manifestations, but could also be employed in the second phase, when the inflammatory and immunologic responses become clinically relevant, due to its anti-inflammatory effects (91).

CONCLUSION

Abnormal ACE2 expression, angiotensin II and angiotensin 1–7 imbalance, and TMPRSS2 androgen-mediated overactivity seem to be key regulators of SARS-CoV-2 infectivity, in accordance with epidemiological observations of hypertension, obesity, and male sex as being major risk factors. Since spironolactone is a long used safe drug that exhibits concurrent actions in the modulation of ACE2 expression that could avoid SARS-CoV-2 cell entry, attenuation of the harms caused by the overexpression of angiotensin II-AT-1 axis, discloses anti-androgenic activity that can decrease viral priming through TMPRSS2 activity, and has anti-inflammatory effects in the lungs, spironolactone seems to be a plausible candidate for the prophylactic and early treatment of SARS-CoV-2.

AUTHOR CONTRIBUTIONS

FC, CW, and AG developed the underlying theories on the present paper, wrote, and reviewed the manuscript in its final format for submission. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

We acknowledge all researchers who have provided insightful and helpful information aiming to overcome the COVID-19 pandemic, as well as all front-line health providers who are directly dealing with COVID-19 infected patients, exposing themselves at risk for this potentially severe infection.

REFERENCES

1. Zhou F, Yu T, Du R, Fan G, Liu Y, Liu Z, et al. Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: a retrospective cohort study. *Lancet*. (2020) 395:1054–62. doi: 10.1016/S0140-6736(20)30566-3
2. Lauer SA, Grantz KH, Bi Q, Jones FK, Zheng Q, Meredith HR, et al. The incubation period of Coronavirus Disease (2019) (COVID-19) from publicly reported confirmed cases: estimation and application. *Ann Intern Med*. (2020) 172:577–82. doi: 10.7326/M20-0504
3. Wu C, Chen X, Cai Y, Xia J, Zhou X, Xu S, et al. Risk factors associated with acute respiratory distress syndrome and death in patients with coronavirus disease 2019. Pneumonia in Wuhan, China. *JAMA Intern Med*. (2020) 180:1–11. doi: 10.1001/jamainternmed.2020.0994
4. Liu W, Tao ZW, Wang L, Yuan ML, Liu K, Zhou L, et al. Analysis of factors associated with disease outcomes in hospitalized patients with 2019 novel coronavirus disease. *Chin Med J*. (2020) 133:1032–8. doi: 10.1097/CM9.0000000000000775
5. Fang L, Karakiulakis G, Roth M. Are patients with hypertension and diabetes mellitus at increased risk for COVID-19 infection? *Lancet Respir Med*. (2020) 8:e21. doi: 10.1016/S2213-2600(20)30116-8
6. Guan WJ, Ni ZY, Hu Y, Liang WH, Ou CQ, He JX, et al. China medical treatment expert group for Covid-19. Clinical characteristics of coronavirus disease 2019 in China. *N Engl J Med*. (2020) 382:1708–20. doi: 10.1101/2020.02.06.20020974
7. Hajifathalian K, Kumar S, Newberry C, Shah S, Fortune B, Krisko T, et al. Obesity is associated with worse outcomes in COVID-19: analysis of Early data from New York City. *Obesity*. (2020). doi: 10.1002/oby.22923. [Epub ahead of print].
8. Kalligeros M, Shehadeh F, Mylona EK, Benitez G, Beckwith CG, Chan PA, et al. Association of obesity with disease severity among patients with coronavirus disease (2019). *Obesity*. (2020) 28:1200–4. doi: 10.1002/oby.22859
9. Palaiodimos L, Kokkinidis DG, Li W, Karamanis D, Ognibene J, Arora S, et al. Severe obesity, increasing age and male sex are independently associated with worse in-hospital outcomes, and higher in-hospital mortality, in a cohort of patients with COVID-19 in the Bronx, New York. *Metabolism*. (2020) 108:154262. doi: 10.1016/j.metabol.2020.154262
10. Goren A, McCoy J, Wambier CG, Vano-Galvan S, Shapiro J, Dhurat R, et al. What does androgenetic alopecia have to do with COVID-19? An insight into a potential new therapy. *Dermatol Ther*. (2020) 1:e13365. doi: 10.1111/dth.13365
11. Goren A, Vaño-Galván S, Wambier CG, McCoy J, Gomez-Zubiaur A, Moreno-Arrones OM, et al. A preliminary observation: male pattern hair loss among hospitalized COVID-19 patients in Spain - a potential clue to the role of androgens in COVID-19 severity. *J Cosmet Dermatol*. (2020) 19:1545–7. doi: 10.1111/jocd.13443
12. Hamming I, Timens W, Bulthuis MLC, Lely AT, Navis GJ, van Goor H. Tissue distribution of ACE2 protein, the functional receptor for SARS coronavirus: a first step in understanding SARS pathogenesis. *J Pathol*. (2004) 203:631–7. doi: 10.1002/path.1570
13. Bourgonje AR, Abdulle AE, Timens W, Hillebrands JL, Navis GJ, Gordijn SJ, et al. Angiotensin-converting enzyme 2 (ACE2), SARS-CoV-2 and the pathophysiology of coronavirus disease 2019 (COVID-19). *J Pathol*. (2020) 251:228–48. doi: 10.1002/path.5471
14. Li Y, Zhou W, Yang L, You R. Physiological and pathological regulation of ACE2, the SARS-CoV-2 receptor. *Pharmacol Res*. (2020) 157:104833. doi: 10.1016/j.phrs.2020.104833
15. Song J, Li Y, Huang X, Chen Z, Li Y, Liu C, et al. Systematic analysis of ACE2 and TMPRSS2 expression in salivary glands reveals underlying transmission mechanism caused by SARS-CoV-2. *J Med Virol*. (2020) 1–11. doi: 10.1002/jmv.26045. [Epub ahead of print].
16. Imai Y, Kuba K, Ohto-Nakanishi T, Penninger JM. Angiotensin-converting enzyme 2 (ACE2) in disease pathogenesis. *Circ J*. (2010) 74:405–10. doi: 10.1253/circj.CJ-10-0045
17. Kuba K, Imai Y, Rao S, Gao H, Guo F, Guan B et al. A crucial role of angiotensin converting enzyme 2 (ACE2) in SARS coronavirus–induced lung injury. *Nat Med*. (2005) 11:875–9. doi: 10.1038/nm1267
18. Hoffmann M, Kleine-Wever H, Kruger N, Muller M, Drotsten C, Pholhlmann S. The novel coronavirus (2019) (2019-nCoV) uses the SARS coronavirus receptor ACE2 and the cellular protease TMPRSS2 for entry in target cells. *Cell*. (2020) 181:1–10. doi: 10.1101/2020.01.31.929042
19. Ortega JT, Serrano ML, Pujol FH, Rangel HR. Role of changes in SARS-CoV-2 spike protein in the interaction with the human ACE2 receptor: an *in silico* analysis. *EXCLI J*. (2020) 19:410–7. doi: 10.17179/excli2020-1167
20. Wambier CG, Goren A. SARS-COV-2 infection is likely to be androgen mediated. *J Am Acad Dermatol*. (2020) 83:308–9. doi: 10.1016/j.jaad.2020.04.032R
21. Pirola CJ, Sookoian S. Estimation of RAAS-Inhibitor effect on the COVID-19 outcome: a meta-analysis. *J Infect*. (2020). doi: 10.1016/j.jinf.2020.05.052. [Epub ahead of print].
22. Cariou B, Hadjadj S, Wargny M, Pichelin M, Al-Salameh A, Allix I, et al. Phenotypic characteristics and prognosis of inpatients with COVID-19 and diabetes: the CORONADO study. *Diabetologia*. (2020) 63:1500–15. doi: 10.1007/s00125-020-05180-x
23. Groß S, Jahn C, Cushman S, Bär C, Thum T. SARS-CoV-2 receptor ACE2-dependent implications on the cardiovascular system: from basic science to clinical implications. *J Mol Cell Cardiol*. (2020) 144:47–53. doi: 10.1016/j.yjmcc.2020.04.031
24. South AM, Diz DI, Chappell MC. COVID-19, ACE2, and the cardiovascular consequences. *Am J Physiol Heart Circ Physiol*. (2020) 318:H1084–90. doi: 10.1152/ajpheart.00217.2020
25. Engeli S, Böhnke J, Gorzelniak K, Janke J, Schling P, Bader M, et al. Weight loss and the renin-angiotensin-aldosterone system. *Hypertension*. (2005) 45:356–62. doi: 10.1161/01.HYP.0000154361.47683.d3
26. Pinheiro TA, Barcala-Jorge AS, Andrade JMO, Pinheiro TA, Ferreira ECN, Crespo TS, et al. Obesity and malnutrition similarly alter the renin-angiotensin system and inflammation in mice and human adipose. *J Nutr Biochem*. (2017) 48:74–82. doi: 10.1016/j.jnutbio.2017.06.008
27. Frantz EDC, Giori IG, Machado MV, Magliano DC, Freitas FM, Andrade MSB, et al. High, but not low, exercise volume shifts the balance of renin-angiotensin system toward ACE2/Mas receptor axis in skeletal muscle in obese rats. *Am J Physiol Endocrinol Metab*. (2017) 313:E473–82. doi: 10.1152/ajpendo.00078.2017
28. Nagase M, Fujita T. Mineralocorticoid receptor activation in obesity hypertension. *Hypertens Res*. (2009) 32:649–57. doi: 10.1038/hr.2009.86
29. Jing F, Mogi M, Horiuchi M. Role of renin-angiotensin-aldosterone system in adipose tissue dysfunction. *Mol Cell Endocrinol*. (2013) 378:23–8. doi: 10.1016/j.mce.2012.03.005
30. Gebhard C, Regitz-Zagrosek V, Neuhauser HK, Morgan R, Klein SL. Impact of sex and gender on COVID-19 outcomes in Europe. *Biol Sex Differ*. (2020) 11:29. doi: 10.1186/s13293-020-00304-9
31. La Vignera S, Cannarella R, Condorelli RA, Torre F, Aversa A, Calogero AE. Sex-specific SARS-CoV-2 mortality: among hormone-modulated ACE2 expression, risk of venous thromboembolism and hypovitaminosis D. *Int J Mol Sci*. (2020) 21:2948. doi: 10.3390/ijms21082948
32. Wambier CG, Goren A, Vaño-Galván S, Ramos PM, Ossimetha A, Nau G, et al. Androgen sensitivity gateway to COVID-19 disease severity. *Drug Dev Res*. (2020). doi: 10.1002/ddr.21688. [Epub ahead of print].
33. Montopoli M, Zumerle S, Vettor R, Rugge M, Zorzi M, Catapano CV, et al. Androgen-deprivation therapies for prostate cancer and risk of infection by SARS-CoV-2: a population-based study ($n = 4532$). *Ann Oncol*. (2020). doi: 10.1016/j.annonc.2020.04.479. [Epub ahead of print].
34. Baig AM, Khaleeq A, Ali U, Syeda H. Evidence of the COVID-19 virus targeting the CNS: tissue distribution, host-virus interaction, and proposed neurotropic mechanisms. *ACS Chem Neurosci*. (2020) 11:995–8. doi: 10.1021/acschemneuro.0c00122
35. Li YC, Bai WZ, Hashikawa T. The neuroinvasive potential of SARS-CoV2 may play a role in the respiratory failure of COVID-19 patients. *J Med Virol*. (2020) 92:552–5. doi: 10.1002/jmv.25728
36. Conde Cardona G, Quintana Pájaro LD, Quintero Marzola ID, Ramos Villegas Y, Moscote Salazar LR. Neurotropism of SARS-CoV 2: mechanisms and manifestations. *J Neurol Sci*. (2020) 412:116824. doi: 10.1016/j.jns.2020.116824

37. Natoli S, Oliveira V, Calabresi P, Maia LF, Pisani A. Does SARS-Cov-2 invade the brain? Translational lessons from animal models. *Eur J Neurol.* (2020). doi: 10.1111/ene.14277. [Epub ahead of print].
38. Helms J, Kremer S, Merdji H, Clere-Jehl R, Schenck M, Kummerlen C, et al. Neurologic features in severe SARS-CoV-2 infection. *N Engl J Med.* (2020) 382:2268–70. doi: 10.1056/NEJMc2008597
39. Lin L, Jiang X, Zhang Z, Huang S, Zhang Z, Fang Z, et al. Gastrointestinal symptoms of 95 cases with SARS-CoV-2 infection. *Gut.* (2020) 69:997–1001. doi: 10.1136/gutjnl-2020-321013
40. Cheung KS, Hung IFN, Chan PPY, Lung KC, Tso E, Liu R, et al. Gastrointestinal manifestations of SARS-CoV-2 infection and virus load in fecal samples from a Hong Kong Cohort: systematic review and meta-analysis. *Gastroenterology.* (2020). doi: 10.1053/j.gastro.2020.03.065. [Epub ahead of print].
41. Xiao F, Tang M, Zheng X, Liu Y, Li X, Shan H. Evidence for gastrointestinal infection of SARS-CoV-2. *Gastroenterology.* (2020) 158:1831–3.e3. doi: 10.1053/j.gastro.2020.02.055
42. Recalcati S. Cutaneous manifestations in COVID-19: a first perspective. *J Eur Acad Dermatol Venereol.* (2020) 34:e212–3. doi: 10.1111/jdv.16387
43. Mungmunpuntipantip R, Wiwanitkit V. COVID-19 and cutaneous manifestations. *J Eur Acad Dermatol Venereol.* (2020) 34:e246. doi: 10.1111/jdv.16483
44. Zhu H, Rhee JW, Cheng P, Waliany S, Chang A, Witteles RM, et al. Cardiovascular complications in patients with COVID-19: consequences of viral toxicities and host immune response. *Curr Cardiol Rep.* (2020) 22:32. doi: 10.1007/s11886-020-01302-4
45. Kim IC, Kim JY, Kim HA, Han S. COVID-19-related myocarditis in a 21-year-old female patient. *Eur Heart J.* (2020) 41:1859. doi: 10.1093/eurheartj/ehaa288
46. Kochi AN, Tagliari AP, Forleo GB, Fassini GM, Tondo C. Cardiac and arrhythmic complications in patients with COVID-19. *J Cardiovasc Electrophysiol.* (2020) 31:1003–8. doi: 10.1111/jce.14479
47. Zeng JH, Liu YX, Yuan J, Wang FX, Wu WB, Li JX, et al. First case of COVID-19 complicated with fulminant myocarditis: a case report and insights. *Infection.* (2020) 10:1–5. doi: 10.1007/s15010-020-01424-5
48. Xu L, Liu J, Lu M, Yang D, Zheng X. Liver injury during highly pathogenic human coronavirus infections. *Liver Int.* (2020) 40:998–1004. doi: 10.1111/liv.14435
49. Lee IC, Huo TI, Huang YH. Gastrointestinal and liver manifestations in patients with COVID-19. *J Chin Med Assoc.* (2020) 83:521–3. doi: 10.1097/JCMA.0000000000000319
50. Musa S. Hepatic and gastrointestinal involvement in coronavirus disease (2019) (COVID-19): what do we know till now? *Arab J Gastroenterol.* (2020) 21:3–8. doi: 10.1016/j.ajg.2020.03.002
51. Fanelli V, Fiorentino M, Cantaluppi V, Gesualdo L, Stallone G, Ronco C, et al. Acute kidney injury in SARS-CoV-2 infected patients. *Crit Care.* (2020) 24:155. doi: 10.1186/s13054-020-02872-z
52. Chu KH, Tsang WK, Tang CS, Lam MF, Lai FM, To KF, et al. Acute renal impairment in coronavirus-associated severe acute respiratory syndrome. *Kidney Int.* (2005) 67:698–705. doi: 10.1111/j.1523-1755.2005.67130.x
53. Abobaker A, Raba AA. Does COVID-19 affect male fertility? *World J Urol.* (2020) 21:1–2. doi: 10.1007/s00345-020-03208-w
54. Cardona Maya WD, Du Plessis SS, Velilla PA. SARS-CoV-2 and the testis: similarity with other viruses and routes of infection. *Reprod Biomed Online.* (2020) 40:763–4. doi: 10.1016/j.rbmo.2020.04.009
55. Klok FA, Kruip MJHA, van der Meer NJM, Arbous MS, Gommers DAMPJ, Kant KM, et al. Incidence of thrombotic complications in critically ill ICU patients with COVID-19. *Thromb Res.* (2020) 191:145–7. doi: 10.1016/j.thromres.2020.04.013
56. Llitjos JF, Leclerc M, Chochois C, Monsallier JM, Ramakers M, Auvray M, et al. High incidence of venous thromboembolic events in anticoagulated severe COVID-19 patients. *J Thromb Haemost.* (2020) 18:1743–6. doi: 10.1111/jth.14869
57. Panigada M, Bottino N, Tagliabue P, Grasselli G, Novembrino C, Chantarangkul V, et al. Hypercoagulability of COVID-19 patients in intensive care unit: a report of thromboelastography findings and other parameters of hemostasis. *J Thromb Haemost.* (2020) 18:1738–42. doi: 10.1111/jth.14850
58. Cui S, Chen S, Li X, Liu S, Wang F. Prevalence of venous thromboembolism in patients with severe novel coronavirus pneumonia. *J Thromb Haemost.* (2020) 18:1421–4. doi: 10.1111/jth.14830
59. Rotzinger DC, Beigelman-Aubry C, von Garnier C, Qanadli SD. Pulmonary embolism in patients with COVID-19: time to change the paradigm of computed tomography. *Thromb Res.* (2020) 190:58–9. doi: 10.1016/j.thromres.2020.04.011
60. Giannis D, Ziogas IA, Gianni P. Coagulation disorders in coronavirus infected patients: COVID-19, SARS-CoV-1, MERS-CoV and lessons from the past. *J Clin Virol.* (2020) 127:104362. doi: 10.1016/j.jcv.2020.104362
61. Georgianos PI, Vaios V, Eleftheriadis T, Zebekakis P, Liakopoulos V. Mineralocorticoid antagonists in ESRD: an overview of clinical trial evidence. *Curr Vasc Pharmacol.* (2017) 15:599–606. doi: 10.2174/1570161115666170201113817
62. Hermidorff MM, Faria Gde O, Amâncio Gde C, de Assis LV, Isoldi MC. Non-genomic effects of spironolactone and eplerenone in cardiomyocytes of neonatal Wistar rats: do they evoke cardioprotective pathways? *Biochem Cell Biol.* (2015) 93:83–93. doi: 10.1139/bcb-2014-0110
63. Cadegiani FA. Can spironolactone be used to prevent COVID-19-induced acute respiratory distress syndrome in patients with hypertension? *Am J Physiol Endocrinol Metab.* (2020) 318:E587–8. doi: 10.1152/ajpendo.00136.2020
64. Nakano S, Kobayashi N, Yoshida K, Ohno T, Matsuoka H. Cardioprotective mechanisms of spironolactone associated with the angiotensin-converting enzyme/epidermal growth factor receptor/extracellular signal-regulated kinases, NAD(P)H oxidase/lectin-like oxidized low-density lipoprotein receptor-1, and Rho-kinase pathways in aldosterone/salt-induced hypertensive rats. *Hypertens Res.* (2005) 28:925–36. doi: 10.1291/hypres.28.925
65. Dieterich HA, Wendt C, Saborowski F. Cardioprotection by aldosterone receptor antagonism in heart failure. Part I. The role of aldosterone in heart failure. *Fiziol Cheloveka.* (2005) 31:97–105. doi: 10.1007/s10747-005-0119-8
66. Taira M, Toba H, Murakami M, Iga I, Serizawa R, Murata S, et al. Spironolactone exhibits direct renoprotective effects and inhibits renal renin-angiotensin-aldosterone system in diabetic rats. *Eur J Pharmacol.* (2008) 589:264–71. doi: 10.1016/j.ejphar.2008.06.019
67. Schjoedt KJ. The renin-angiotensin-aldosterone system and its blockade in diabetic nephropathy: main focus on the role of aldosterone. *Dan Med Bull.* (2011) 58:B4265.
68. Kong EL, Zhang JM, An N, Tao Y, Yu WF, Wu FX. Spironolactone rescues renal dysfunction in obstructive jaundice rats by upregulating ACE2 expression. *J Cell Commun Signal.* (2019) 13:17–26. doi: 10.1007/s12079-018-0466-2
69. Takeda Y, Zhu A, Yoneda T, Usukura M, Takata H, Yamagishi M. Effects of aldosterone and angiotensin II receptor blockade on cardiac angiotensinogen and angiotensin-converting enzyme 2 expression in Dahl salt-sensitive hypertensive rats. *Am J Hypertens.* (2007) 20:1119–24. doi: 10.1016/j.amjhyper.2007.05.008
70. Zhu A, Yoneda T, Demura M, Karashima S, Usukura M, Yamagishi M, et al. Effect of mineralocorticoid receptor blockade on the renal renin-angiotensin system in Dahl salt-sensitive hypertensive rats. *J Hypertens.* (2009) 27:800–5. doi: 10.1097/HJH.0b013e328325d861
71. Keidar S, Gamliel-Lazarovich A, Kaplan M, Pavlotzky E, Hamoud S, Hayek T, et al. Mineralocorticoid receptor blocker increases angiotensin-converting enzyme 2 activity in congestive heart failure patients. *Circ Res.* (2005) 97:946–53. doi: 10.1161/01.RES.0000187500.24964.7A
72. Te Riet L, van Esch JH, Roks AJ, van den Meiracker AH, Danser AH. Hypertension: renin-angiotensin-aldosterone system alterations. *Circ Res.* (2015) 116:960–75. doi: 10.1161/CIRCRESAHA.116.303587
73. Hamming I, Cooper ME, Haagmans BL, Hooper NM, Korstanje R, Osterhaus AD, et al. The emerging role of ACE2 in physiology and disease. *J Pathol.* (2007) 212:1–11. doi: 10.1002/path.2162
74. Patel S, Rauf A, Khan H, Abu-Izneid T. Renin-angiotensin-aldosterone (RAAS): the ubiquitous system for homeostasis and pathologies. *Biomed Pharmacother.* (2017) 94:317–25. doi: 10.1016/j.biopha.2017.07.091

75. Sert M, Tetiker T, Kirim S. Comparison of the efficiency of anti-androgenic regimens consisting of spironolactone, Diane 35, and cyproterone acetate in hirsutism. *Acta Med Okayama*. (2003) 57:73–6. doi: 10.18926/AMO/32820
76. Steelman SL, Brooks JR, Morgan ER, Patanelli DJ. Anti-androgenic activity of spironolactone. *Steroids*. (1969) 14:449–50. doi: 10.1016/S0039-128X(69)80007-3
77. Broulik PD, Stárka L. Antiandrogenic and antirenotropic effect of spironolactone. *Endokrinologie*. (1976) 68:35–9.
78. Vecchiola A, Fuentes CA, Solar I, Lagos CF, Opazo MC, Muñoz-Durango N, et al. Eplerenone implantation improved adipose dysfunction averting RAAS activation and cell division. *Front Endocrinol*. (2020) 11:223. doi: 10.3389/fendo.2020.00223
79. Feraco A, Armani A, Mammi C, Fabbri A, Rosano GM, Caprio M. Role of mineralocorticoid receptor and renin-angiotensin-aldosterone system in adipocyte dysfunction and obesity. *J Steroid Biochem Mol Biol*. (2013) 137:99–106. doi: 10.1016/j.jsbmb.2013.02.012
80. Ji WJ, Ma YQ, Zhang X, Zhang L, Zhang YD, Su CC, et al. Inflammatory monocyte/macrophage modulation by liposome-entrapped spironolactone ameliorates acute lung injury in mice. *Nanomedicine*. (2016) 11:1393–406. doi: 10.2217/nnm-2016-0006
81. Ji WJ, Ma YQ, Zhou X, Zhang YD, Lu RY, Guo ZZ, et al. Spironolactone attenuates bleomycin-induced pulmonary injury partially via modulating mononuclear phagocyte phenotype switching in circulating and alveolar compartments. *PLoS ONE*. (2013) 8:e81090. doi: 10.1371/journal.pone.0081090
82. Rafatian N, Westcott KV, White RA, Leenen FH. Cardiac macrophages and apoptosis after myocardial infarction: effects of central MR blockade. *Am J Physiol Regul Integr Comp Physiol*. (2014) 307:R879–87. doi: 10.1152/ajpregu.00075.2014
83. Zhang L, Hao JB, Ren LS, Ding JL, Hao LR. The aldosterone receptor antagonist spironolactone prevents peritoneal inflammation and fibrosis. *Lab Invest*. (2014) 94:839–50. doi: 10.1038/labinvest.2014.69
84. Ozacmak HS, Ozacmak VH, Barut F, Arasli M, Ucan BH. Pretreatment with mineralocorticoid receptor blocker reduces intestinal injury induced by ischemia and reperfusion: involvement of inhibition of inflammatory response, oxidative stress, nuclear factor κB, and inducible nitric oxide synthase. *J Surg Res*. (2014) 191:350–61. doi: 10.1016/j.jss.2014.04.040
85. Kato Y, Kamiya H, Koide N, Odkhuu E, Komatsu T, Dagvadorj J, et al. Spironolactone inhibits production of proinflammatory mediators in response to lipopolysaccharide via inactivation of nuclear factor-κB. *Immunopharmacol Immunotoxicol*. (2014) 36:237–41. doi: 10.3109/08923973.2014.921690
86. Lieber GB, Fernandez X, Mingo GG, Jia Y, Caniga M, Gil MA, et al. Mineralocorticoid receptor antagonists attenuate pulmonary inflammation and bleomycin-evoked fibrosis in rodent models. *Eur J Pharmacol*. (2013) 718:290–8. doi: 10.1016/j.ejphar.2013.08.019
87. Fraccarollo D, Galuppo P, Schraut S, Kneitz S, van Rooijen N, Ertl G, et al. Immediate mineralocorticoid receptor blockade improves myocardial infarct healing by modulation of the inflammatory response. *Hypertension*. (2008) 51:905–14. doi: 10.1161/HYPERTENSIONAHA.107.100941
88. Lacombe B, Morel M, Margottin-Goguet F, Ramirez BC. Specific inhibition of HIV infection by the action of spironolactone in T cells. *J Virol*. (2016) 90:10972–80. doi: 10.1128/JVI.01722-16
89. Verma D, Thompson J, Swaminathan S. Spironolactone blocks Epstein-Barr virus production by inhibiting EBV SM protein function. *Proc Natl Acad Sci USA*. (2016) 113:3609–14. doi: 10.1073/pnas.1523686113
90. Yartaş Dumanli G, Dilken O, Ürkmez S. Use of spironolactone in SARS-CoV-2 ARDS patients. *Turk J Anaesthesiol Reanim*. (2020) 48:254–55. doi: 10.5152/TJAR.2020.569
91. Alifano M, Alifano P, Forgez P, Iannelli A. Renin-angiotensin system at the heart of COVID-19 pandemic. *Biochimie*. (2020) 174:30. doi: 10.1016/j.biochi.2020.04.008

Conflict of Interest: AG is the CEO of the company Applied Biology Inc (Irvine, CA, USA). CW is part of the medical board of the company Applied Biology Inc (Irvine, CA, USA).

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Public Health

OPINION
published: 28 July 2020
doi: 10.3389/fpubh.2020.00393

Improving Non-specific Immunity to Coronavirus Disease (COVID-19) by the Novelty, Diversity, and Quantity of Antigen

Patrice Boucher[1]* *and Roger Boucher*[2]

[1] *Software and Information Technology Engineering Department, École de Technologie Supérieure, Montréal, QC, Canada,*
[2] *Médicentre, LaSalle, QC, Canada*

Keywords: coronavirus, COVID-19, natural killer cells, vaccination, non-specific immunity, pandemic crisis

THE OBJECTIVE: SAVE LIVES

The health crisis caused by the COVID-19 pandemic continues to claim thousands of lives around the world. The current challenge for the scientific community, along with governments, is to quickly find solutions to save lives and limit the consequences of the crisis.

The scientific community is currently tackling the problem primarily by aiming to develop a vaccine to enable the body to develop an antigen-specific immune response to COVID-19. This approach requires time-consuming studies in order to understand the underlying properties of COVID-19 and to deploy the vaccine (1), which is very challenging to achieve at pandemic speed (2). Moreover, it could be compromised by mutations in the COVID-19 genome (3).

However, it is essential to note that the problem could be approached from at least one other front for more rapid deployment in a pandemic context. Too little consideration has been devoted to the roles of the non-specific immune response via natural killer (NK) cells, which continue to be neglected in research into vaccines (4).

AN ALTERNATIVE APPROACH: EXPLOITING THE ACTION OF NK CELLS

NK cells are involved in innate resistance that is not antigen-specific, but these cells also play roles in adaptive immunity by favoring the development of antigen-specific T helper (Th) type 1 cells by producing IFN-γ and IL-2 (5–7). In other words, a better NK effector cell response [via the production of IFN-γ and the exocytosis of cytotoxic granules (4)] directly contributes to virus neutralization and to the efficiency by which specific antigens are developed at the time of infection. During an infection, NK cells also specialize as memory NK cells, which mediate protection against a second infection by the same pathogen (4, 8). The ability of these memory cells to mediate protection against other pathogens should therefore be further investigated.

Since the beginning of the pandemic, children have presented the highest resistance to COVID-19, but this resistance gradually decreases with age (9). In the first phase of infection, the innate immune system of children typically neutralizes the virus, but this is ineffective in the older population, which commonly lacks NK cells during this phase (10). The depletion of NK cells correlates with the severity of infections (10, 11). Therefore, the perspective of enhancing the response of the non-specific immune system should be seriously investigated in the context of this pandemic.

OPEN ACCESS

Edited by:
Zisis Kozlakidis, International Agency For Research On Cancer (IARC), France

Reviewed by:
Kohei Fujita, National Hospital Organization Kyoto Medical Center, Japan
Xiaojiong Jia, Harvard Medical School, United States

***Correspondence:**
Patrice Boucher
patrice.boucher.qc@gmail.com

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Public Health

Received: *12 May 2020*
Accepted: *06 July 2020*
Published: *28 July 2020*

Citation:
Boucher P and Boucher R (2020) Improving Non-specific Immunity to Coronavirus Disease (COVID-19) by the Novelty, Diversity, and Quantity of Antigen. Front. Public Health 8:393. doi: 10.3389/fpubh.2020.00393

IMPROVING IMMUNITY TO COVID-19

Horowitz et al. (4) demonstrated that vaccination enhances NK cell effector responses in an antigen-specific manner for a fairly long period of time (their tests were conducted after about 4 months), which agrees with the documented properties of memory NK cells (8). Hence, if multiple vaccines enhance the NK effector response with diverse memory NK cells, this suggests that they may significantly contribute to (i) the neutralization of the COVID-19 pathogen, and (ii) the development of specific antibodies at the time of infection.

Children experience many novel antigens via vaccines, influenza, and other environmental pathogens. The speed by which NK cells neutralize the pathogen, as seen in children, is the main factor limiting its propagation in the body. The literature shows that the response of the immune system can be trained by episodic infections. We propose the hypothesis that the *novelty, diversity, and quantity of antigen could play a key role in training the non-specific immune system to evoke a fast, efficient response to pathogens*.

In order to test this hypothesis, we aim to reproduce the experience of children with many diverse and potentially novel antigens, before analyzing the effects of such experiences on the immune system. The complexity of this task should require numerous research projects, giving rise to a new avenue for research in immunology. Nevertheless, this could be investigated in the first instance by the revaccination of older people with a set of vaccines administered to young children. For the novelty of antigen, the use of vaccines that are new to a target population (e.g., existing vaccines in foreign countries) could also be studied. In such a study, a group of vaccinated subjects and a group of demographically equivalent non-vaccinated subjects should be periodically tested for COVID-19. If vaccination effectively improves non-specific immunity, the infected subjects of the vaccinated group should be less severely infected on average (including lower morbidity and more asymptomatic cases) than the infected subjects in the non-vaccinated group. Here, an improvement in non-specific immunity is expected, in particular, if (i) an increased concentration of NK cells is observed and lasts for several months/years; (ii) these NK cells (including primitive NK cells and memory NK cells developed in response to vaccination) are effective against new antigens; and (iii) they accelerate the development of antibodies.

It is worth mentioning that vaccines have shown to be less effective on older people, who are subject to *immunosenescence* (12). In spite of the adverse events reported, e.g., by the Vaccine Adverse Event Reporting System (VAERS) in the U.S., several vaccines have proven to be secure and relevant for adults and older people including, for example, the vaccines for measles, mumps, and rubella (13); influenza (14); pneumococcal conjugate with PCV13 and PCV23 (15); tetanus toxoid, reduced diphtheria, and acellular pertussis (16, 17); herpes zoster (18); and acute upper respiratory tract infection with the Bacillus Calmette-Guérin vaccine (19, 20), which is also under study for improving immunity to COVID-19 (21).

Yu et al. (22) analyzed the effect of childhood vaccinations on cross-reactivity against SARS-CoV in mice by evaluating the ability of T cells to recognize the SARS-CoV antigen in vaccinated compared to unvaccinated mice. They did not observe a significant difference between the two groups. Their conclusion was that "the reduced symptoms among children infected by SARS-CoV may be caused by other factors [than vaccination]"; however, this ignores the effects of the non-specific immune system via NK cells. First, as suggested by Horowitz et al. (4), vaccines given to children could be rather beneficial by improving the response of NK cells. Second, given the interspecies differences in NK cell activity (23), we do not know whether NK cells neutralize the virus prior to the development of specific antibodies.

CONCLUSION

We propose the hypothesis that the *novelty, diversity, and quantity of antigen could play a key role in training the non-specific immune system for a fast, efficient response to pathogens*. If true, it is expected that vaccination with existing vaccines could help vulnerable populations to fight the virus more effectively. This hypothesis should be seriously considered since:

- It is supported by the recently documented action of the non-specific immune system via NK cells, and is coherent with clinical observations (despite the fact that they are still not sufficient to validate it). For example, this hypothesis would explain why children are more resistant to COVID-19 and why immunity gradually decreases with age.
- This could mean that thousands of lives can be saved quickly.

It could be addressed empirically by a statistical analysis of the occurrence of COVID-19 and severity in a targeted population in which we administer a set of existing vaccines. This approach could also be tested in resource-restricted settings. Most countries around the world, including developing countries, currently have access to many low-cost vaccines (24).

Given the urgency of the situation, we recommend that the scientific community, in cooperation with governments, rapidly investigate this hypothesis. Such a study would be quick, easy, and inexpensive to perform, with little risk to the population as the effects of existing vaccines are already known and validated.

AUTHOR CONTRIBUTIONS

RB initiated the proposal ideas. PB developed the ideas and prepared the proposal with the support of RB. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

The authors wish to acknowledge the editor and the reviewers of Frontiers for their constructive comments that contribute to the relevance and the quality of the paper; Virginie Grenier-Deschênes, Christine Boucher, and Proof-Reading-Service.com for proofreading.

REFERENCES

1. Le TT, Andreadakis Z, Kumar A, Roman RG, Tollefsen S, Saville M, et al. The COVID-19 vaccine development landscape. *Nat Rev Drug Discov*. (2020) 19:305–6. doi: 10.1038/d41573-020-00073-5
2. Lurie N, Saville M, Hatchett R, Halton J. Developing Covid-19 vaccines at pandemic speed. *N Engl J Med*. (2020) 382:1969–73. doi: 10.1056/NEJMp2005630
3. van Dorp L, Acman M, Richard D, Shaw LP, Ford CE, Ormond L, et al. (2020). Emergence of genomic diversity and recurrent mutations in SARS-CoV-2. *Infect Genet Evol*. 83:104351. doi: 10.1016/j.meegid.2020.104351
4. Horowitz A, Behrens RH, Okell L, Fooks AR, Riley EM. NK cells as effectors of acquired immune responses: effector CD4+ T cell-dependent activation of NK cells following vaccination. *J Immunol*. (2010) 185:2808–18. doi: 10.4049/jimmunol.1000844
5. Trinchieri G. Natural killer cells wear different hats: effector cells of innate resistance and regulatory cells of adaptive immunity and of hematopoiesis. *Semin Immunol*. (1995) *7*:83–8. doi: 10.1006/smim.1995.0012
6. Romagnani S. Induction of TH1 and TH2 responses: a key role for the 'natural' immune response? *Immunol Today*. (1992) 13:379–81.
7. Scott P, Trinchieri G. The role of natural killer cells in host-parasite interactions. *Curr Opin Immunol*. (1995) 7:34–40.
8. Sun JC, Beilke JN, Lanier LL. Adaptive immune features of natural killer cells. *Nature*. (2009) 457:557–61. doi: 10.1038/nature07665
9. Ludvigsson JF. Systematic review of COVID-19 in children shows milder cases and a better prognosis than adults. *Acta Paediatr*. (2020) 109:1088–95. doi: 10.1111/apa.15270
10. Zheng M, Gao Y, Wang G, Song G, Liu S, Sun D, et al. Functional exhaustion of antiviral lymphocytes in COVID-19 patients. *Cell Mol Immunol*. (2020) 17:533–5. doi: 10.1038/s41423-020-0402-2
11. Ebbo M, Gérard L, Carpentier S, Vély F, Cypowyj S, Farnarier C, et al. Low circulating natural killer cell counts are associated with severe disease in patients with common variable immunodeficiency. *EBioMedicine*. (2016) 6:222–30. doi: 10.1016/j.ebiom.2016.02.025
12. Goronzy J, Weyand C. Understanding immunosenescence to improve responses to vaccines. *Nat Immunol*. (2013) 14:428–36. doi: 10.1038/ni.2588
13. Sukumaran L, McNeil MM, Moro PL, Lewis PW, Winiecki SK, Shimabukuro TT. Adverse events following measles, mumps, and rubella vaccine in adults reported to the vaccine adverse event reporting system (VAERS), 2003-2013. *Clin Infect Dis*. (2015) 60:e58–65. doi: 10.1093/cid/civ061
14. Manzoli L, Salanti G, De Vito C, Boccia A, Ioannidis JP, Villari P. Immunogenicity and adverse events of avian influenza A H5N1 vaccine in healthy adults: multiple-treatments meta-analysis. *Lancet Infect Dis*. (2009) 9:482–92. doi: 10.1016/S1473-3099(09)70153-7
15. Kraicer-Melamed H, O'Donnell S, Quach C. The effectiveness of pneumococcal polysaccharide vaccine 23 (PPV23) in the general population of 50 years of age and older: a systematic review and meta-analysis. *Vaccine*. (2016) 34:1540–50. doi: 10.1016/j.vaccine.2016.02.024
16. Moro PL, Yue X, Lewis P, Haber P, Broder K. Adverse events after tetanus toxoid, reduced diphtheria toxoid and acellular pertussis (Tdap) vaccine administered to adults 65 years of age and older reported to the Vaccine Adverse Event Reporting System (VAERS), 2005-2010. *Vaccine*. (2011) 29:9404–8. doi: 10.1016/j.vaccine.2011.05.100
17. Weston WM, Friedland LR, Wu X, Howe B. Vaccination of adults 65 years of age and older with tetanus toxoid, reduced diphtheria toxoid and acellular pertussis vaccine Boostrix(®): results of two randomized trials. *Vaccine*. (2012) 30:1721–8. doi: 10.1016/j.vaccine.2011.12.055
18. Gnann JW Jr. Vaccination to prevent herpes zoster in older adults. *J Pain*. (2008) 9(Suppl. 1):S31–6. doi: 10.1016/j.jpain.2007.10.007
19. Wardhana, Datau EA, Sultana A, Mandang VV, Jim E. The efficacy of Bacillus Calmette-Guerin vaccinations for the prevention of acute upper respiratory tract infection in the elderly. *Acta Medica Indonesia*. (2011) 43:185–90.
20. Turnbull FM, McIntyre PB, Achat HM, Wang H, Stapledon R, Gold M, Burgess MA. National study of adverse reactions after vaccination with bacille Calmette-Guérin. *Clin Infect Dis*. (2002) 34:447–53. doi: 10.1086/338462
21. Redelman-Sidi G. Could BCG be used to protect against COVID-19? *Nat Rev Urol*. (2020) 17:316–7. doi: 10.1038/s41585-020-0325-9
22. Yu Y, Jin H, Chen Z, Qingling LY, Ma YJ, Sun XL, et al. Children's vaccines do not induce cross reactivity against SARS-CoV. *J Clin Pathol*. (2007) 60:208–11. doi: 10.1136/jcp.2006.038893
23. Haley PJ. Species differences in the structure and function of the immune system. *Toxicology*. (2003) 188:49–71. doi: 10.1016/s0300-483x(03)00043-x
24. World Health Organization. *Global and Regional Immunization Profile. Who Vaccine-Preventable Disease Monitoring System*. (2019).

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Medicine

OPINION
published: 28 July 2020
doi: 10.3389/fmed.2020.00429

Healthcare Transformation in the Post-Coronavirus Pandemic Era

Abdul Rahman Jazieh[1]* *and Zisis Kozlakidis*[2]

[1] *Department of Oncology, King Abdullah International Medical Research Center, King Saud bin Abdulaziz University for Health Sciences, Riyadh, Saudi Arabia,* [2] *Laboratory Services and Biobanking, International Agency for Research on Cancer, World Health Organization, Lyon, France*

Keywords: COVID-19, healthcare transformation, ethics, technological innovation, coronavirus

The effects of the coronavirus disease 2019 (COVID-19) pandemic globally are striking as it impacts greatly the social, political, economic, and healthcare aspects of many countries. The toll of this pandemic quantified with human lives and suffering (1), the psychosocial impact (2), and the economic slowdown (3) constitute strong reasons to translate experiences into actionable lessons, not simply to prevent similar future crises, but rather to improve the whole spectrum of population health and healthcare delivery. This is the third coronavirus (CoV) outbreak of international concern in 20 years, after the severe acute respiratory syndrome (SARS-CoV) and the Middle-East respiratory syndrome (MERS-CoV), in addition to other viral outbreaks such as Zika virus and Ebola virus over the last decade. It becomes clear that infectious diseases should be considered among the most important health hazards that we will need to continue facing in the foreseeable future (4). Thus, the transformation of various aspects at the individual as well as the societal and governmental levels seems inevitable.

The COVID-19 pandemic has become a reality check for many aspects of healthcare systems, especially regarding their overall readiness. Public health surveillance programs and available infrastructures were shown as not consistently optimal (5–7). Additionally, healthcare systems appeared unable to absorb and manage sudden and persistent pressures on their workload especially in the settings of acute care. Even though contingency plans were often in place, healthcare systems seemed unable to cope with the sudden, intense surge in demand (8, 9). From a policy perspective, potential delay(s) in committing to major decisions, such as lockdown measures, in an "epidemiologically timely fashion" could significantly impact downstream healthcare outcomes (10, 11). The latter is of particular importance, as healthcare challenges in one country should be considered both an internal and a potentially global challenge, at least for infectious diseases (12, 13). Finally, the speed at which a global public health issue translated into a financial downturn, affecting many different industries, was underestimated (3, 14).

The COVID-19 pandemic acts as a transformation catalyst, accelerating the implementation and adoption of changes in public health interventions. Thus, a new model of healthcare delivery emerges with more emphasis on preventive measures, remote care, and substantial technological dependence. However, these are juxtaposed against ongoing technical challenges to meet the surge capacity in laboratory testing, the fast-tracked implementation of new technologies, the mental health concerns, the ethical concerns on the potential rationing of insufficient resources, and the protection of privacy and personal data during times of crises. Taking the former into account, the following aspects seem likely to emerge as most affected in the post-COVID-19 era.

SHIFTING GREATER PATIENT NUMBERS TO REMOTE CARE

Remote care or telehealth services were already used in emergencies, crises, and routine care previously (15, 16). During the COVID-19 pandemic, their wider utilization has accelerated. Telehealth services have now been used in the large-scale screening of patients prior to their visit

OPEN ACCESS

Edited by:
Longxiang Su,
Peking Union Medical College Hospital (CAMS), China

Reviewed by:
Tauqeer Hussain Mallhi,
Al Jouf University, Saudi Arabia
Tarek A. Ahmad,
Bibliotheca Alexandrina, Egypt

***Correspondence:**
Abdul Rahman Jazieh
jazieha@ngha.med.sa

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: *09 May 2020*
Accepted: *03 July 2020*
Published: *28 July 2020*

Citation:
Jazieh AR and Kozlakidis Z (2020) Healthcare Transformation in the Post-Coronavirus Pandemic Era. Front. Med. 7:429. doi: 10.3389/fmed.2020.00429

and triage assessment, in the routine monitoring of patients at home, for remote clinical encounters, or supervising patient care by off-site experts (17–19). It is likely that a significant portion of such services will remain telehealth-based post COVID-19, e.g., the remote monitoring and management of greater numbers of patients, as it provides higher convenience and better patient-centered care, thus partially addressing the healthcare system flow rate and capacity challenges.

This has been observed in mental healthcare as well, where the pandemic became a catalyst for the implementation of online therapy and e-health tools in routine practice, following more than two decades of many brilliant, but mostly failed, attempts (20, 21). Imperatives dominating the field, e.g., that "the clinician/patient therapeutic alliance can only be established face-to-face," in spite of research showing the opposite (22), are being resolved. It is likely that once mental healthcare institutions have developed the capabilities post COVID-19 of serving their patients *via* different digital technologies, there is little reason for them to give all of these up, in view of the advantages they have experienced over an extended period of crisis response (23). A future "blended approach" is likely to emerge, where e-mental-health solutions occupy a greater part of routine services. Additionally, the currently developed expertise can be used in expanding a wider public e-mental-health approach, utilizing not only guided but also fully self-guided interventions, such as self-help apps or online therapeutic modules (24). The latter could also be tested and eventually applied in settings and countries with scarce mental health resources, where such need has been previously identified (25), as a positive post-COVID-19 long-term outcome.

This system evolution is likely to serve as an adjunct for the gradual adoption of further new technologies, for example, the use of drones as delivery vehicles for critical supplies, robotics, the widespread 3D-printing of healthcare-related items, and smartphone-enabled monitoring of patient adherence to treatments (26, 27).

IMPROVED EMPHASIS ON SURVEILLANCE SYSTEMS AND DATA ANALYSIS

The speed by which SARS-CoV-2 spread globally highlights once more that the need for reliable and representative surveillance systems for infectious diseases remains as acute as ever. Public health surveillance for infectious diseases uses reported positive results from sentinel clinical laboratories or laboratory networks to survey the presence of specific microbial agents that constitute a threat to public health in a given population (28). However, the continuing rationalization of public health costs has led to the consolidation of a number of clinical microbiology laboratories involving a shift toward laboratory amalgamation. Through this consolidation activity, an operational model emerged with large centralized clinical laboratories performing on one central platform and one or several distal laboratories dealing locally only with urgent analyses (29, 30). It would be informative to see if this reduction in the number of small clinical laboratories and the aggregation of the remaining ones conditioned or not the ability to detect epidemiological changes in the context of COVID-19.

Therefore, the routine use of big data and artificial intelligence approaches to model crises and to identify and understand the weaknesses of existing systems (close to real-time) would be necessary in order to strengthen existing structures. Mobile-enabled technologies can now be deployed *en masse* to monitor quarantined individuals and to trace exposed individuals in a timely and accurate fashion within regions and/or countries, as in the cases of South Korea and Taiwan (31). These are some of the new tools likely to move further into the public health sphere and support the understanding in an interconnected and hypercomplex global environment. The necessity for international collaboration and sharing of information between competent healthcare authorities during crises has been highlighted many times previously, as well as the rapid deployment of specialist teams on the ground, and this is likely to be strengthened even further post COVID-19 (32, 33).

Any such changes would need to be accompanied by a greater public awareness of the health systems, new and/or better tools, and their potential implementations in order to combat infectious disease outbreaks. As such, the interaction with social media and behavioral science is likely to be used extensively for health promotion, education, and mass communications (34). However, the pandemic has also highlighted that poor health literacy among the general population is an underestimated public health problem globally (35). Improving public health literacy is now essential as it might help people to grasp the reasons behind the recommendations and reflect on outcomes of their various possible actions, especially in the context of resource-restricted settings (36).

DEVELOPMENT OF LEGISLATIVE, POLITICAL, AND HEALTHCARE MANAGEMENT SYSTEMS

While the COVID-19 outbreak accelerated many of the above processes, there still remain challenges, including, for example, credentialing, licensing, reimbursement, and issues related to technology, security, privacy, safety, and litigations (37–39). More specifically, in the ethical field and from an individual perspective, the collection and availability of vast amounts of information regarding people (e.g., *via* geo-tagged social networks) makes full data anonymization ineffective in protecting the identity of the data source, making it only more difficult, yet still feasible *via* the use of advanced systems and triangulation, to (re)identify individuals (40). As such, the ethical imperative of transparency with regard to the dangers of downstream data linkage and inadvertent individual identification should be upheld (41). From a population-level perspective, if systems are designed to be entirely reliant on anonymous data in order to protect data contributors, they might not work very well-either, as the element of information accountability and, hence, transparency is affected. Especially in the case of humanitarian emergencies, and certainly

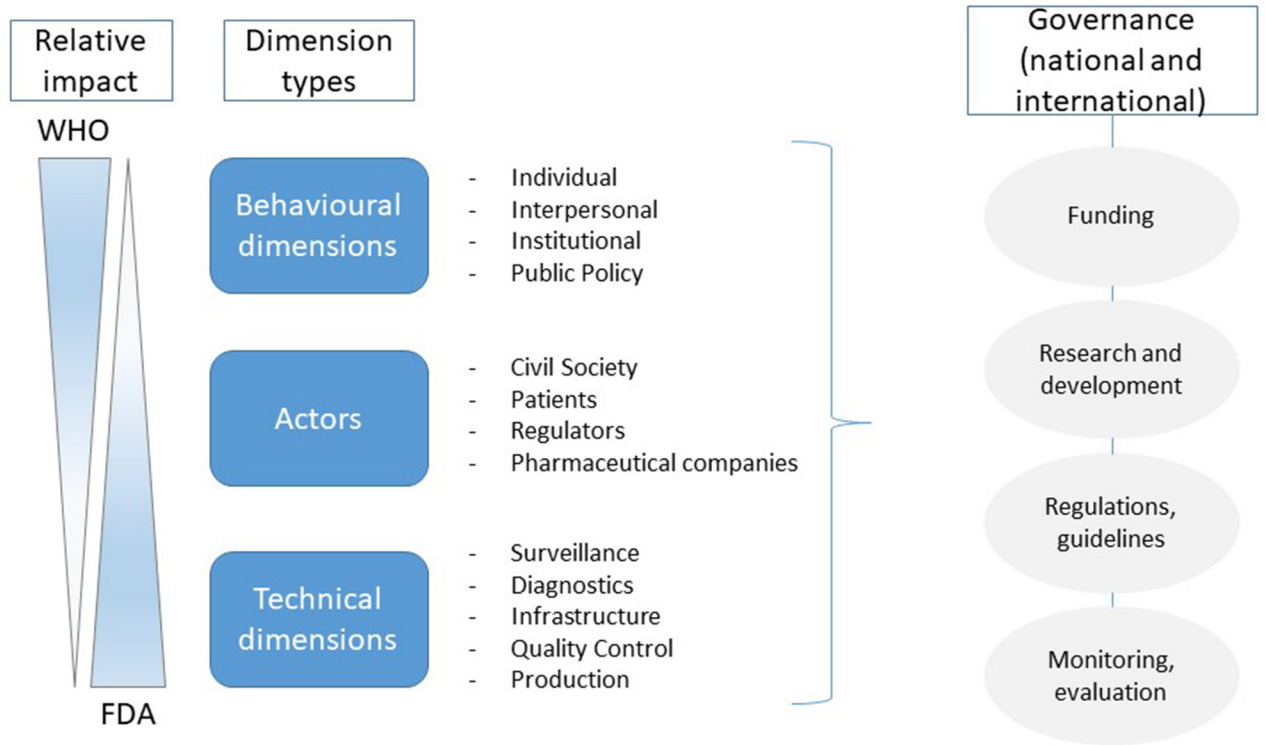


FIGURE 1 | The simplified relative impact of the organizations mentioned in the manuscript (adapted) (49). This schematic representation defines the different influence domains of the main actors during the pandemic and the need for a coordinated international approach for future responses to coronavirus disease 2019 (COVID-19).

communicable disease outbreaks, anonymous information is current best practice, but cannot be considered as the ethical panacea (42).

It should be noted that public health ethics differs from clinical ethics in that it requires giving priority to promoting the common good over protecting individual autonomy (43). This ethical contrast becomes even greater in resource-restricted settings during public health emergencies, where overwhelmed healthcare systems might instigate the rationing of staff and/or medical supplies, with distressing decision-making, such as who receives life support (44).

One of the defining aspects of the current pandemic was the unprecedented levels of misinformation, conspiracy theories, and rumors reproduced by lay and social media related to COVID-19; these can only be counterproductive in the fight against the current epidemic, both in the short and long term. Perhaps, this is an outcome of the pandemic taking place during the "social media age." (45). The WHO responded to the "infodemic" releasing a statement and suppressing several such measures advocated online and in social media, which are not effective in the treatment of COVID-19, and has done so ongoingly (46). In terms of responses, social media platforms have responded to the majority of the social media posts rated false by fact-checkers by removing them or attaching various warnings. However, as the number of English-language fact-checks rose more than 900% from January to March, outpacing the available fact-checking resources, misinformation has almost certainly grown even faster (47). Consistency in the public health messaging as well as increased funding dedicated to fact-checking seems to be needed as the immediate first step.

It seems inevitable that post COVID-19, there will be a review of policies, guidelines, and regulations relating to individuals' rights and the implementation of drastic public health measures, such as prolonged quarantine measures as well as the governance of new technologically driven solutions within healthcare (relative impact shown in **Figure 1**). Compulsory "public health-triggered" powers are currently justified under a common legal and ethical standard, taking into account the risk of the pathogen to the individual and the general population, the incidence rate and transmission mode of the pathogen, the effectiveness of available public health interventions, and the availability and type of clinical treatments. In particular, in emerging crises, such as in the case of COVID-19 when the science is uncertain, the adoption of the "precautionary principle" is reasonable to ensure public safety. It is expected that post COVID-19, a number of these measures will be evaluated on their timing and effectiveness, whether the nature of the measures and their implementation was proportionate to the risk, and whether the legal assessments of the partial scientific evidence were successful (48).

DEVELOPMENT OF COMMUNICATION TECHNOLOGY-BASED APPROACHES

The transformation in healthcare would not be possible if it is not associated with technological innovations in communication, machine learning, and transportation. The expansion of Medicare telehealth coverage amidst the pandemic is a major step in the right direction (50) as well as the increased delivery of healthcare closer to home for chronic neurological patients (51). However, concerns about security remain as not all publicly available tools for videoconferencing comply with internationally accepted standards of confidentiality. This security concern, for example, the phenomenon of Zoom-bombing, applies as much to patients as it does to medical professionals delivering the new remote services. Therefore, technology-empowered approaches must take all necessary steps to safeguard the privacy of their participants.

The silver lining for the post-COVID-19 era is the realization that a significant portion of healthcare activities in the wider sense can be improved by technologically empowered approaches, and some can even be done remotely equally as effectively. For example, for some postoperative follow-ups, phone visits are not necessarily inferior to in-person visits in terms of patient satisfaction, complications, and adverse events (52). Where there needs to be an increased emphasis is the investigation of how technologies can be utilized earlier and/or better in order to provide added flexibility to the responsiveness of the healthcare system in times of crisis. Existing guidelines have supported part of this perspective (53). However, there has been a noticeable struggle to shift the focus of healthcare systems to tackle the current emergency resulting in potential response timelags (54).

DEVELOPMENT OF FINANCIAL MODELS TO SUPPORT SCIENTIFIC RESEARCH, COOPERATION, AND CRISIS PREPAREDNESS

COVID-19 led simultaneously to two opposite consequences on laboratory medicine activities. On the one hand, microbiology departments faced a huge increase in their diagnostic activities related to the afflux of COVID-19 suspected patients (55). On the other hand, activities of clinical laboratories not directly related to COVID-19 dropped significantly, including for instance cancer services, which had to adapt to a different, remote-based service model (17, 18). A similar picture was also observed at the institutional/hospital level, with a drop of routine activity (56), and the acute need for reallocation of staff and services (57). Considering these factors, COVID-19 has changed the healthcare business models of basic academic health sciences, public health surveillance, and the industry. The efficient collaboration within informal networks comprising clinical laboratories servicing consortia of hospitals, academic groups, and test manufacturers (forged through previous recent outbreaks and/or operational consolidations) represented a key element in the European response against COVID-19 and in supporting acute clinical and international needs (e.g., utilization of the existing COMBACTE Network) (58).

Thus, it is likely that because of their quick mobilization and response times to the clinical needs, further global activities such as those within the Innovative Medicines Initiative (IMI) framework will be strengthened, hopefully maintaining the breadth of creative approaches. The urgency of the COVID-19 situation forced major healthcare providers to respond often without the ability for a full discussion of the financial costs involved in those emergency responses. However, the scale of investment needed for combatting COVID-19 is certainly ambitious and a key consideration for the immediate future. As such, new public private partnerships are vital, whether this involves drug, vaccine, and/or test development. Unlocking additional financing sources, acknowledging the imperative to link financial returns to the providers of capital, and creating profitable, sustainable financing structures will be central in developing new financial models to support scientific research, cooperation, and crisis preparedness (59).

CONCLUSION

The COVID-19 outbreak serves as a reminder that proactive planning for healthcare emergencies as well as an intensified commitment to global public health preparedness remains necessary. The lessons learned on the limitations of extant healthcare systems and their capacity to respond to infectious disease epidemics in the 21st century should be considered, enabling the transformation of future healthcare. In addition, the realization that technologically empowered solutions can be implemented and work well-should constitute the benchmark for the greater integration of such technologies as part of routine healthcare design and provision. Optimal outcomes can be attained where both patients and healthcare providers become active participants in this process. However, for that to be achieved, ethical, regulatory, and legal concerns that emerged during this pandemic need to be addressed. The current global experiences lay the foundation for a significant post-COVID-19 healthcare transformation, so that systems can better prepare to address the next global threat(s) of the 21st century.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

REFERENCES

1. World Health Organization. *Coronavirus Disease 2019 (COVID-19) Situation Report – 109*. (2020) Available online at: https://www.who.int/docs/default-source/coronaviruse/situation-reports/20200508covid-19-sitrep-109.pdf?sfvrsn=68f2c632_6 (accessed May 9, 2020).
2. Carvalho P, Moreira MM, de Oliveira M, Landim J, Neto M. The psychiatric impact of the novel coronavirus outbreak. *Psychiatr Res.* (2020) 286:112902. doi: 10.1016/j.psychres.2020.112902
3. *Coronavirus: The world Economy at risk.* (2020). Available online at: http://www.oecd.org/economic-outlook/ (accessed May 9, 2020).
4. Mattiuzzi C, Lippi G. Which lessons shall we learn from the 2019 novel coronavirus outbreak? *Ann Transl Med.* (2020) 8:48. doi: 10.21037/atm.2020.02.06
5. Paterlini M. On the front lines of coronavirus: the Italian response to Covid-19. *BMJ*. (2020) 368:m1065. doi: 10.1136/bmj.m1065
6. Nkengasong J. China's response to a novel coronavirus stands in stark contrast to the 2002 SARS outbreak response. *Nat Med*. (2020) 26:310–1. doi: 10.1038/s41591-020-0771-1
7. Kolie D, Delamou A, van de Pas R, Dioubaté N, Bouedouno P, Beavogui AH, et al. Never let a crisis go to waste: postEbola agenda-setting for health system strengthening in Guinea. *BMJ Global Health*. (2019) 4:e001925. doi: 10.1136/bmjgh-2019-001925
8. Hick JL, Hanfling D, Wynia MK, Pavia AT. *Duty to Plan: Health Care, Crisis Standards of Care, and Novel Coronavirus SARS-CoV-2. NAM Perspectives*. Discussion paper. Washington, DC: National Academy of Medicine. (2020)
9. Kandel N, Chungong S, Omaar A, Xing J. Health security capacities in the context of COVID-19 outbreak: an analysis of international health regulations annual report data from 182 countries. *Lancet*. (2020) 395:1047–53. doi: 10.1016/S0140-6736(20)30553-5
10. Hafiz H, Oei S-Y, Ring DM, Shnitser N. *Regulating in Pandemic: Evaluating Economic and Financial Policy Responses to the Coronavirus Crisis*. Boston College Law School Legal Studies Research Paper. (2020). Available online at: https://ssrn.com/abstract=3555980 (accessed May 9, 2020).
11. Carter DP, May PJ. Making sense of the U.S. COVID-19 pandemic response: A policy regime perspective. *Administ Theory Praxis*. (2020) 45:265–77. doi: 10.1080/10841806.2020.1758991
12. Ravi SJ, Snyder MR, Rivers C. Review of international efforts to strengthen the global outbreak response system since the 2014–16 West Africa Ebola Epidemic. *Health Policy Plann.* (2019) 34:47–54. doi: 10.1093/heapol/czy102
13. Rodríguez-Morales AJ, MacGregor K, Kanagarajah S, Patel D, Schlagenhauf P. Going global - Travel and the 2019 novel coronavirus. *Travel Med Infect Dis*. (2020) 33:101578. doi: 10.1016/j.tmaid.2020.101578
14. Ayittey FK, Ayittey MK, Chiwero NB, Kamasah JS, Dzuvor C. Economic impacts of Wuhan 2019-nCoV on China and the world. *J Med Virol*. (2020) 92:473–5. doi: 10.1002/jmv.25706
15. Langabeer JR, Gonzalez M, Alqusairi D, Champagne-Langabeer T, Jackson A, Mikhail J, et al. Telehealth-enabled emergency medical services program reduces ambulance transport to urban emergency departments. *West J Emerg Med*. (2016) 17:713–20. doi: 10.5811/westjem.2016.8.30660
16. Lurie N, Carr BG. The role of telehealth in the medical response to disasters. *JAMA Intern Med*. (2018) 178:745–6. doi: 10.1001/jamainternmed.2018.1314
17. Hollander JE, Carr BG. Virtually perfect? Telemedicine for Covid-19. *N Engl J Med*. (2020) 382:1679–81. doi: 10.1056/NEJMp2003539
18. Zhai Y, Wang Y, Zhang M, Gittell JH, Jiang S, Chen B, et al. From isolation to coordination: how can telemedicine help combat the COVID-19 outbreak? *medRxiv [Preprint]*. (2020). doi: 10.1101/2020.02.20.20025957
19. Greenhalgh T, Wherton J, Shaw S, Morrison C. Video consultations for covid-19. *BMJ.* (2020) 368:m998. doi: 10.1136/bmj.m998
20. Mohr DC, Riper H, Schueller SM. A solution-focused research approach to achieve an implementable revolution in digital mental health. *JAMA Psychiat*. (2018) 75:113–4. doi: 10.1001/jamapsychiatry.2017.3838
21. Vis C, Mol M, Kleiboer A, Bührmann L, Finch T, Smit J, et al. Improving implementation of eMental health for mood disorders in routine practice: systematic review of barriers and facilitating factors. *JMIR Ment Health*. (2018) 5:e20. doi: 10.2196/mental.9769
22. Berger T. The therapeutic alliance in internet interventions: a narrative review and suggestions for future research. *Psychother Res*. (2017) 27:511–24. doi: 10.1080/10503307.2015.1119908
23. Tuerk PW, Keller SM, Acierno R. Treatment for anxiety and depression via clinical videoconferencing: evidence base and barriers to expanded access in practice. *Focus*. (2019) 16:363–9. doi: 10.1176/appi.focus.20180027
24. Wind TR, Rijkeboer M, Andersson G, Riper H. The COVID-19 pandemic: The 'black swan' for mental health care and a turning point for e-health. *Internet interventions*. (2020) 20:100317. doi: 10.1016/j.invent.2020.100317
25. Christiani Y, Setiawan A. Internet-based treatment of depression in Indonesia. *Lancet Psychiatr*. (2018) 5:688–9. doi: 10.1016/S2215-0366(18)30261-X
26. Ullah H, Nair NG, Moore A, Nugent C, Muschamp P, Cuevas M. 5G communication: an overview of vehicle-to-everything, drones, and healthcare use-cases. *IEEE Access*. (2019) 7:37251–68. doi: 10.1109/ACCESS.2019.2905347
27. Story A, Aldridge WA, Smith CM, Garber E, Hall J, Ferenando G, et al. Smartphone-enabled video-observed versus directly observed treatment for tuberculosis: a multicentre, analyst-blinded, randomised, controlled superiority trial. *Lancet*. (2019) 393:1216–24. doi: 10.1016/S0140-6736(18)32993-3
28. Zeng D, Chen H, Castillo-Chavez C, Thurmond M. Clinical laboratory data for biosurveillance. In: Zeng D, Chen H, Castillo-Chavez C, Lober WB, Thurmond M, editors. *Infectious Disease Informatics and Biosurveillance*. New York, NY: Springer (2011). p. 67–87.
29. Vandenberg O, Kozlakidis Z, Schrenzel J, Struelens MJ, Breuer J. Control of infectious diseases in the era of European clinical microbiology laboratory consolidation: new challenges and opportunities for the patient and for public health surveillance. *Front Med*. (2018) 5:15. doi: 10.3389/fmed.2018.00015
30. Vandenberg O, Durand G, Hallin M, Diefenbach A, Gant V, Murray P, et al. Consolidation of clinical microbiology laboratories and introduction of transformative technologies. *Clin Microbiol Rev*. (2020) 33:e00057-19. doi: 10.1128/CMR.00057-19
31. Wang CJ, Ng CY, Brook RH. Response to COVID-19 in Taiwan. *JAMA*. (2020) 323:1341–2. doi: 10.1001/jama.2020.3151
32. Ogden NH, Wilson JRU, Richardson DM, Hui C, Davies SJ, Kumschick S, et al. Emerging infectious diseases and biological invasions: a call for a One Health collaboration in science and management. *R Soc Open Sci*. (2019) 6:181577. doi: 10.1098/rsos.181577
33. Kyhlstedt M, Andersson SW. Diagnostic and digital solutions to address the COVID-19 pandemic: the need for international collaboration to close the gap. *Health Policy Technol.* (2020) 9:126–8. doi: 10.1016/j.hlpt.2020.04.010
34. Lunn PD, Belton CA, Lavin C, McGowan FP, Timmons S, Robertson DA. Using behavioral science to help fight the coronavirus. *J Behav Public Administ.* (2020) 3:1. doi: 10.30636/jbpa.31.147
35. Paakkari L, Okan O. COVID-19: health literacy is an underestimated problem. *Lancet Public Health*. (2020) 5:e249–50. doi: 10.1016/S2468-2667(20)30086-4
36. Nguyen HC, Nguyen MH, Do BN, Tran CQ, Nguyen TT, Pham KM. et al. People with suspected COVID-19 symptoms were more likely depressed and had lower health-related quality of life: The potential benefit of health literacy. *J Clin Med.* (2020) 9:965. doi: 10.3390/jcm9040965
37. Alami H, Gagnon MP, Wootton R, Fortin JP, Zanaboni P. Exploring factors associated with the uneven utilization of telemedicine in Norway: A mixed methods study. *BMC Med Inform Decis Mak*. (2017) 17:180. doi: 10.1186/s12911-017-0576-4
38. Shaw S, Wherton J, Vijayaraghavan S, Morris J, Bhattacharya S, Hanson P, et al. Advantages and limitations of virtual online consultations in a NHS acute trust: the VOCAL mixed-methods study. *Health Serv Deliv Res*. (2018) 6:21. doi: 10.3310/hsdr06210
39. Donaghy E, Atherton H, Hammersley V, McNeilly H, Bikker A, Robbins L, et al. Acceptability, benefits, and challenges of video consulting: a qualitative study in primary care. *Br J Gen Pract*. (2019) 69:E586–94. doi: 10.3399/bjgp19X704141
40. Cecaj A, Mamei M, Zambonelli F. Re-identification and information fusion between anonymized CDR and social network data. *J Ambient Intellig Human Comput.* (2016) 7:83–96. doi: 10.1007/s12652-015-0303-x
41. Garattini C, Raffle J, Aisyah DN, Sartain F, Kozlakidis Z. Big data analytics, infectious diseases and associated ethical impacts. *Philos Technol.* (2019) 32:69–85. doi: 10.1007/s13347-017-0278-y

42. Coltart CEM, Hoppe A, Parker M, Dawson L, Amon JJ, Simwinga M, et al. Ethical considerations in global HIV phylogenetic research. *Lancet HIV*. (2018) 5:e656–66. doi: 10.1016/S2352-3018(18)30134-6
43. Chia T, Oyeniran OI. Human health versus human rights: an emerging ethical dilemma arising from coronavirus disease pandemic. *Ethics Med Public Health*. (2020) 11:100511. doi: 10.1016/j.jemep.2020.100511
44. White DB, Katz MH, Luce JM, Lo B. Who should receive life support during a public health emergency? Using ethical principles to improve allocation decisions. *Ann Intern Med*. (2009) 150:132–8. doi: 10.7326/0003-4819-150-2-200901200-00011
45. Smith GD, Ng F, Li WHC. COVID-19: Emerging compassion, courage and resilience in the face of misinformation and adversity. *J Clin Nursing*. (2020) 29:1425. doi: 10.1111/jocn.15231
46. World Health Organization. *Novel Coronavirus(2019-nCoV) Situation Report*. (2020). Available Online at: https://www.who.int/docs/default-source/coronaviruse/situation-reports/20200202-sitrep-13-ncov-v3.pdf (accessed February 13, 2020).
47. Brennen JS, Simon FM, Howard PN, Nielsen RK. Types, sources, and claims of Covid-19 misinformation. *Reuters Institute*. (2020). Available online at: https://reutersinstitute.politics.ox.ac.uk/types-sources-and-claims-covid-19-misinformation
48. Gostin LO, Hodge JG. US emergency legal responses to novel coronavirus: balancing public health and civil liberties. *JAMA*. (2020) 323:1131–2. doi: 10.1001/jama.2020.2025
49. Bloom G, Merrett GB, Wilkinson A, Lin V, Paulin S. Antimicrobial resistance and universal health coverage. *BMJ Global Health*. (2017) 2:e000518. doi: 10.1136/bmjgh-2017-000518
50. *White House Expands Medicare Telehealth Services Amid COVID-19*. Available online at: https://www.medscape.com/viewarticle/927075 (accessed May 9, 2020).
51. Bloem BR, Dorsey ER, Okun MS. The coronavirus disease 2019 crisis as catalyst for telemedicine for chronic neurological disorders. *JAMA Neurol*. (2020). doi: 10.1001/jamaneurol.2020.1452. [Epub ahead of print].
52. Thompson JC, Cichowski SB, Rogers RG, Qeadan F, Zambrano J, Wenzl C, et al. Outpatient visits versus telephone interviews for postoperative care: a randomized controlled trial. *Int Urogynecol J*. (2019) 30:1639–46. doi: 10.1007/s00192-019-03895-z
53. World Health Organization. *Strengthening and Adjusting Public Health Measures Throughout the COVID-19 Transition Phases. Policy Considerations for the WHO European Region*. (2020). Available online at: http://www.euro.who.int/__data/assets/pdf_file/0018/440037/Strength-AdjustingMeasuresCOVID19-transition-phases.pdf?ua=1 (accessed May 9, 2020).
54. Tanne JH, Hayasaki E, Zastrow M, Pulla P, Smith P, Rada AG. Covid-19: how doctors and healthcare systems are tackling coronavirus worldwide. *BMJ*. (2020) 368:m1090. doi: 10.1136/bmj.m1090
55. Roser M, Ritchie H, Ortiz-Ospina E, Hasell J. Coronavirus pandemic (COVID-19). (2020). Published online at OurWorldInData.org. Available online at: https://ourworldindata.org/coronavirus (accessed July 1, 2020).
56. Tartari F, Guglielmo A, Fuligni F, Pileri A. Changes in emergency service access after spread of COVID19 across Italy. *J Eur Acad Dermatol Venereol*. (2020). doi: 10.1111/jdv.16553. [Epub ahead of print].
57. Grimm CA. *Hospital Experiences Responding to the COVID-19 Pandemic: Results of a National Pulse Survey March 23–27, 2020*. U.S. Department of Health and Human Services. Office of Inspector General. Available online at: https://www.oig.hhs.gov/oei/reports/oei-06-20-00300.pdf
58. *Combacte: Combatting Bacterial Resistance in Europe*. Available online at: https://www.combacte.com/
59. Krech R, Kickbusch I, Franz C, Wells N. Banking for health: the role of financial sector actors in investing in global health. *BMJ Glob Health*. (2018) 3(Suppl 1):e000597. doi: 10.1136/bmjgh-2017-000597

Disclaimer: Where authors are identified as personnel of the International Agency for Research on Cancer/WHO, the authors alone are responsible for the views expressed in this article and they do not necessarily represent the decisions, policy, or views of the International Agency for Research on Cancer/WHO.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 28 July 2020
doi: 10.3389/fimmu.2020.01784

Contriving Multi-Epitope Subunit of Vaccine for COVID-19: Immunoinformatics Approaches

Rong Dong[1,2,3†], *Zhugang Chu*[4], *Fuxun Yu*[3] *and Yan Zha*[1,2,3*]

[1] Department of Biomedicine, Guizhou University School of Medicine, Guiyang, China, [2] Department of Nephrology, Guizhou Provincial People's Hospital, Guiyang, China, [3] NHC Key Laboratory of Pulmonary Immunological Diseases (Guizhou Provincial People's Hospital), Guiyang, China, [4] Department of Urinary Surgery, Guizhou Provincial People's Hospital, Guiyang, China

COVID-19 has recently become the most serious threat to public health, and its prevalence has been increasing at an alarming rate. The incubation period for the virus is ∼1–14 days and all age groups may be susceptible to a fatality rate of about 5.9%. COVID-19 is caused by a novel single-stranded, positive (+) sense RNA beta coronavirus. The development of a vaccine for SARS-CoV-2 is an urgent need worldwide. Immunoinformatics approaches are both cost-effective and convenient, as *in silico* predictions can reduce the number of experiments needed. In this study, with the aid of immunoinformatics tools, we tried to design a multi-epitope vaccine that can be used for the prevention and treatment of COVID-19. The epitopes were computed by using B cells, cytotoxic T lymphocytes (CTL), and helper T lymphocytes (HTL) base on the proteins of SARS-CoV-2. A vaccine was devised by fusing together the B cell, HTL, and CTL epitopes with linkers. To enhance the immunogenicity, the β-defensin (45 mer) amino acid sequence, and pan-HLA DR binding epitopes (13aa) were adjoined to the N-terminal of the vaccine with the help of the EAAAK linker. To enable the intracellular delivery of the modeled vaccine, a TAT sequence (11aa) was appended to C-terminal. Linkers play vital roles in producing an extended conformation (flexibility), protein folding, and separation of functional domains, and therefore, make the protein structure more stable. The secondary and three-dimensional (3D) structure of the final vaccine was then predicted. Furthermore, the complex between the final vaccine and immune receptors (toll-like receptor-3 (TLR-3), major histocompatibility complex (MHC-I), and MHC-II) were evaluated by molecular docking. Lastly, to confirm the expression of the designed vaccine, the mRNA of the vaccine was enhanced with the aid of the Java Codon Adaptation Tool, and the secondary structure was generated from Mfold. Then we performed *in silico* cloning. The final vaccine requires experimental validation to determine its safety and efficacy in controlling SARS-CoV-2 infections.

Keywords: immunoinformatics, epitope prediction, COVID-19, SARS-CoV-2, vaccine

OPEN ACCESS

Edited by:
Rashika El Ridi,
Cairo University, Egypt

Reviewed by:
Ahmad Karkhah,
Babol University of Medical Sciences, Iran
James Lyons-Weiler,
Institute for Pure and Applied Knowledge, United States

****Correspondence:***
Yan Zha
zhayan72@126.com

†ORCID:
Rong Dong
orcid.org/0000-0003-2372-9833

Specialty section:
This article was submitted to Vaccines and Molecular Therapeutics, a section of the journal Frontiers in Immunology

Received: *27 March 2020*
Accepted: *03 July 2020*
Published: *28 July 2020*

Citation:
Dong R, Chu Z, Yu F and Zha Y (2020) Contriving Multi-Epitope Subunit of Vaccine for COVID-19: Immunoinformatics Approaches. Front. Immunol. 11:1784. doi: 10.3389/fimmu.2020.01784

INTRODUCTION

In December 2019, COVID-19, caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was first discovered in China and has rapidly spread across the world. As of 12:00 noon on June 4, a total of 6,392,319 confirmed cases of COVID-19 have been reported globally, including 383,318 deaths. The prevalence of the disease has been increasing at an alarming rate.

There were 1,849,852 cases in the United States, 555,383 in Brazil, 431,715 in Russia, 281,270 in the United Kingdom, and 3,275,736 in a number of other countries (1).

The incubation period for the virus is ∼1–14 days, and all age groups are susceptible to a fatality rate of about 5.9%. The most common clinical manifestations are low-grade fever, dry cough, fatigue, and gastrointestinal symptoms (2). About half of all patients with COVID-19 develop shortness of breath, and severe cases may rapidly develop SARS, septic shock, difficult-to-correct metabolic acidosis, and coagulation disorders (3). COVID-19 may also affect other organs, most commonly the heart and kidneys (4–6). Some patients may have mild symptoms, without fever, and may recover after 1–4 weeks (7). Other patients may show signs of serious illness and some may die; however, most patients show favorable progress (8). Male individuals with the disease and aged patients have the worst prognosis. In children, the disease is relatively mild (9).

COVID-19 is caused by a novel single-stranded, positive (+) sense RNA beta coronavirus, which is a pathogen of the *Coronaviridae* family, named SARS-CoV-2 (10). The full-length genome sequences revealed that SARS-CoV-2 has the greatest genetic similarity to bat coronavirus, ∼45–90% similarity to severe acute respiratory syndrome-related coronavirus (SARSr-CoV), and a smaller similarity of 20–60% to the Middle East respiratory syndrome-related coronavirus (MERS-CoV) (10). Thus, a bat might be the original host of SARS-CoV-2, but the intermediate host remains undiscovered (10).

The genes of SARS-CoV-2 encode structural proteins and non-structural proteins. Four structural proteins are absolutely vital for viral assembly and invasion of SARS-CoV-2. Spike protein homotrimers constitute the spikes on the viral surface, and these spikes are responsible for attachment to host cells by binding to their receptors (10). The M protein has three transmembrane domains, which determine the shape of the virion, facilitate membrane curvature, and bind to the nucleocapsid. The *E* protein plays an important role in virion assembly and release, as well as involved in viral pathogenesis. The *N* protein has two different domains, both of which bind to the viral RNA genome via totally different mechanisms. In addition, some reports have shown that non-structural proteins are essential for the replication of coronaviruses (10).

Vaccination is a vital tool for the control and elimination of the virus, and the development of a vaccine for SARS-CoV-2 remains an urgent need (11). Traditional methods of vaccine development are time-consuming and very labor-intensive (12). The realm of immunoinformatics tools considers the mechanism of the host immune response to yield additional methodologies in the design of vaccine against diseases are cost-effective and convenient, as *in silico* predictions can reduce the number of experiments needed (13, 14). Dozens of studies have generated epitope-based peptide vaccine of SARS-CoV-2. Baruah and Bose (15) used immunoinformatics tools to discover cytotoxic T lymphocyte (CTL) and B cell epitopes for the spike protein of SARS-CoV-2. Then, Abraham et al. developed a multi-epitope vaccine that was designed using immunoinformatics tools that potentially trigger both $CD4^+$ and $CD8^+$ T-cell immune responses (16).

Although there are many vaccines generated by immunoinformatics tools, most of these are based on spike protein. The spike protein is responsible for attachment to host cells by binding to angiotensin-converting enzyme 2 (ACE2) (17). A vaccine based on the spike protein could induce antibodies to block SARS-COV binding and fusion or neutralize virus infection (18). But there are still many obstacles, spike protein-based SARS vaccine may induce harmful immune responses that cause liver damage of the vaccinated animals (19). Other virus proteins are considered as the candidates for designing vaccine with protective and less harmful immune responses (20). Vaccine-based on structural and non-structural proteins of the virus is revealed potential vaccine inducing protective immune responses (20, 21). Pandey et al. reported the more scientifically rigorous strategy of multi-epitope subunits based on multiple proteins against parasitic and viral diseases, such as malaria, visceral leishmaniasis, and HIV (22–24). In this present, we employed immunoinformatics to predict multiple immunogenic proteins from the SARS-CoV-2 proteome and thereby design a multi-epitope vaccine. These proteins included non-structural and structural sequences of SARS-CoV-2, their reference sequences were retrieved from the National Center for Biotechnology Information (NCBI) database.

MATERIALS AND METHODS

Retrieving COVID-19 Protein Sequences

The proteins of the SARS-CoV-2 have been reported and reference could get from NCBI (25, 26). The reference sequences of SARS-CoV-2 proteins were retrieved from NCBI Protein Database (https://www.ncbi.nlm.nih.gov/protein) and accession numbers in **Table 1**, then we stored the reference sequences as a FASTA data type. The proteins with <100 amino acid sequences which are too short to predict epitopes were excluded, the remaining proteins were used for further analysis.

Identifying Antigenicity of Protein Sequences

VaxiJen is the first server for alignment-independent prediction of protective antigens, which overcome the limitations of alignment-dependent methods (27). To identify the potential antigenicity of SARS-CoV-2 proteins, an online prediction server, VaxiJen v2.0 (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html) was used to predict the antigenic values of each protein (28). This identification was applied according to the default parameters of the server. Proteins having antigenicity were sorted according to an antigenic score of $\geq$ 0.5 (Threshold for this model is 0.5) and were selected for further structural modeling (27).

Structural Modeling of SARS-COV-2 Proteins

There are no available experimental structures of SARS-COV-2 proteins, Phyre 2 provide model regions trough a new ab initio folding simulation with no detectable homology (29). The SARS-CoV-2 proteins were modeled by Phyre 2 server (http://www.sbg.bio.ic.ac.uk/phyre2/). Because the SARS-COV-2 proteins with no

TABLE 1 | Details and antigenicity of SARS-CoV-2 proteins.

No.	Accession number[a]	Protein	Amino acids	Antigenic value[b]
1	YP_009724395.1	ORF7a protein	121aa	0.6755
2	YP_009724396.1	ORF8 protein	121aa	0.6392
3	YP_009725305.1	nsp9	113aa	0.6292
4	YP_009725302.1	nsp6	290aa	0.5668
5	YP_009725299.1	nsp3	1945aa	0.5538
6	YP_009725310.1	endoRNAse	346aa	0.5436
7	YP_009724391	ORF3a protein	275aa	0.541
8	YP_009724393.1	Membrane glycoprotein	222aa	0.5184
9	YP_009724397.2	Nucleocapsid phosphoprotein	419aa	0.5133
10	YP_009725295.1	ORF1a polyprotein	4405aa	0.4813
11	YP_009725300.1	nsp4	500aa	0.4759
12	YP_009724390.1	Surface glycoprotein	1273aa	0.4707
13	YP_009724389.1	ORF1ab polyprotein	7096aa	0.4624
14	YP_009725297.1	Leader protein	180aa	0.4497
15	YP_009725309.1	3′-to-5′ exonuclease	527aa	0.4219
16	YP_009725308.1	Helicase	601aa	0.4219
17	YP_009725307.1	RNA-dependent RNA polymerase	932aa	0.4123
18	YP_009725306.1	nsp10	139aa	0.4091
19	YP_009725304.1	nsp8	198aa	0.4063
20	YP_009725298.1	nsp2	638aa	0.4043
21	YP_009725301.1	3C-like proteinase	306aa	0.4037
22	YP_009725311.1	2′-O-ribose	298aa	0.3917
23	YP_009724394.1	ORF6 protein	61aa	0.5719
24	YP_009725296.1	ORF7b protein	43aa	0.5505
25	YP_009725255.1	ORF10 protein	38aa	0.622
26	YP_009725312.1	nsp11	13aa	0.2878
27	YP_009724392.1	Envelope protein	75aa	0.6243

[a] *The accession number is the National Center for Biotechnology Information (NCBI) reference sequence number.*
[b] *The antigenic value threshold was > 0.5000.*

detectable homology protein to finish the modeling, we chose the intensive search and output the accurate alignment by the alignment of hidden Markov models.

ModRefiner was used by the GalaxyRefine server (http://galaxy.seoklab.org /cgi-bin/submit.cgi?type=REFINE) (30). The structure assessment was performed by the SWISS-MODEL workspace (https://swissmodel.expasy.org/assess) (31). The three dimensional (3D) models were used for the conformational (discontinuous) B-cell epitope predictions while the sequences were utilized in linear B-cell and T-cell epitope predictions.

Prediction of CTL Epitopes

NetCTL-1.2 is demonstrated to have a high predictive performance (32). The NetCTL 1.2 server (http://www.cbs.dtu.dk/services/NetCTL/) was applied to predict CTL epitopes for the SARS-CoV-2 at the threshold value of 0.75 with high sensitivity and specificity (32). To cover ∼90% of the world's population, three supertypes (A2, A3, and B7) were selected based on artificial neural networks, to predict MHC class I binding epitopes (33). The best candidates for the SARS-CoV-2 vaccine construction were sorted for further prediction, based on a half-maximal inhibitory concentration (IC50) < 500 nm and an integrated score. The IC50 < 500 nm represents epitope has a high affinity to receptor. The integrated score indicated the transporter of antigenic peptides (TAP) transport efficiency, class I binding, and proteasomal cleavage prediction (34–36). Then the specific Treg epitopes were screened and excluded by the EpiToolKit (https://epivax.com/).

Prediction of Helper T Lymphocyte (HTL) Epitopes

For MHC class II T cell epitope predictions, The Immune Epitope Database server predicted binders based on the percentile rank or MHC binding affinity (37). The Immune Epitope Database server (IEDB; http://tools.iedb.org/mhcii/) was used to predict helper T lymphocyte (HTL) epitopes (37). We chose the combinatorial approach which recommended by IEDB to predict HTL epitopes. The combinatorial approach combined NN-align, SMM-align, CombLib, Sturniolo, and NetMHCIIpan methods (38–42). The 17 alleles of the human leukocyte antigen (HLA) were selected for the prediction at α and β chains, separately (43). For final construction, epitopes were selected based on their scores (low scores indicated favorable binding), the release of interferon-gamma (IFN-γ), induction of emergent properties, and the IC50 < 500 nm.

Prediction of IFN-γ Inducing Epitopes

The IFN-γ cytokine makes a major contribution to antiviral mechanisms. It excites both native and specific immune responses by activating macrophages and natural killer cells (44). Further, IFN-γ augments the response of MHC to antigens. The IFN-γ epitope server (http://crdd.osdd.net/raghava/ifnepitope/scan.php) was used to recognize IFN-γ epitopes (45). We entered the HTL epitopes with low scores into the IFN-γ epitope server. Positive IFN-γ induction was predicted based on the support vector machine (SVM) hybrid approach. The final HTL epitopes were determined based on IFN-γ induction and MHC Class II binding, both of which facilitate the stimulation of T-helper cells (46).

Prediction of Line and Conformational B Cell Epitopes

The ABCpred (http://crdd.osdd.net/raghava/abcpred/) and BepiPred linear epitope prediction (http://tools.iedb.org/bcell/result/) servers were utilized to predict linear B cell epitopes. The ABCpred server is based on an artificial neural network (ANN) (47, 48). The linear B cell epitopes of the SARS-CoV-2 protein were predicted at a threshold of 0.5. The BepiPred linear epitope prediction server is based on seven methods: (a) Bepipred-1.0 Linear Epitope Prediction; (b) BepiPred-2.0: Sequential B cell Epitope Predictor; (c) Chou and Fasman beta-turn prediction; (d) Emini surface accessibility scale; (e) Karplus and Schulz flexibility scale; and the (f) Kolaskar and Tongaonkar antigenicity scale (49–54). We used these seven methods separately to predict the average threshold. The

overlap between ABCpred and BepiPred severs was selected to determine the candidate epitopes for the SARS-CoV-2 vaccine construction (55).

Unlike T-cell epitopes that are linear continuous stretches of residues, B-cell epitopes are generally conformational (discontinuous) (56). In this study, the ElliPro servers (http://tools.iedb.org/ellipro/) were applied to predict the conformational B-cells epitopes (57). The server predicts epitopes based on PI (Protrusion Index) value. The epitope with PI = 0.9 would include 90% of residues with 10% being outside the ellipsoid, discontinues B-cells epitopes with the top PI value was selected for vaccine designing (57).

Multi-Epitope Subunit Vaccine Design

To develop the final vaccine, epitopes determined by various immunoinformatics software were linked together with the aid of separate linkers. The CTL epitopes were linked by the AAY linker, HTL epitopes by the GPGPG linker, and B cells were linked by the KK linker (48, 58, 59). To increase the vaccine immunogenicity, the β-defensin (45 mer) amino acid sequence was adjoined to the N-terminal of the vaccine with the help of the EAAAK linker (60). The β-defensin peptides provoke innate immunity cells and recruit naive T cells through the chemokine receptor-6 (CCR-6) (61). The pan-HLA DR binding epitopes (13aa) as well as added to the N-terminal of the vaccine with the aid of the same linker (59). The pan-HLA DR binding epitopes in vaccine construct facilitating binding to many different types of mouse and human MHC-II alleles to induce CD4-helper cell responses (59). To enable the intracellular delivery of the modeled vaccine, a TAT sequence (11aa) was appended to C-terminal (62). Linkers (AYY, KK, and GPGPG) play vital roles in producing an extended conformation (flexibility), protein folding, and separation of functional domains, and therefore, make the protein structure more stable (59).

Prediction of Allergenicity, Antigenicity

The allergenic proteins induce a harmful immune response, allergenicity of the vaccine should be non-allergic (63). The non-allergic character of the vaccine sequence was evaluated by the AlgPred server (http://www.imtech.res.in/raghava/algpred/) (63). We predicted allergenicity of vaccine sequences choosing a hybrid approach (SVMc+IgE epitope+ARPs BLAST+MAST) with the highest accuracy and sensitivity (63).

The Vaxijen v2.0 server (http://www.ddgpharmfac.net/vaxijen/VaxiJen/VaxiJen.html) was applied to evaluate the antigenicity of the vaccine (27). The antigenicity prediction method was solely based on the physicochemical properties of proteins without recourse to sequence alignment. The precision rate of the server ranged from 70 to 89%.

Immune Simulations

To determine immune response profile of this multi-epitope vaccine, computational immune simulations were performed by the C-ImmSim online server at (http://kraken.iac.rm.cnr.it/C-IMMSIM/) (64). The C-ImmSim utilizes the Celada-Seiden model for describing both humoral and cellular profiles of a mammalian immune system against designed vaccine. As per the literature, three injections were administrated at different intervals of 1 month. The simulation was performed with default parameters. The vaccine sequence was administered 4 weeks apart. The simulation volume was 1,000, simulation steps was 1,000, random seed was 12,345, and the vaccine injection with no LPS (64).

Prediction of Various Physicochemical Properties

The ProtParam tool (http://web.expasy.org/protparam/) was used to evaluate the physicochemical properties of the final vaccine protein (65). The physicochemical properties included the number of amino acids, molecular weight, theoretical isoelectric point (pI), amino acid composition, atomic composition, formula, extinction coefficients, estimated half-life, instability index, aliphatic index, and grand average of hydropathicity (GRAVY) (66). The molecular weight and theoretical pI were computed by user-entered sequences. The amino acid and atomic compositions were self-explanatory. The extinction coefficient of a protein was based on information about its amino acid composition. The instability index of a protein indirectly indicated the stability of the protein. If the computed instability index of protein was <40, it was regarded as a stable protein, while values >40 were regarded as unstable. *In vivo* half-life evaluation of proteins was based on the principle of the "N-end rule." Furthermore, GRAVY is a measurement of the hydrophobic nature of the protein, which is calculated by determining the total hydropathy of all amino acids divided by the number of amino acid residues in the protein.

To avoid inducing pathogenic priming and autoimmunity, the sequence homology of the final vaccine to human protein was screened by BLASTp online server (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (67). An ideal vaccine should have non-sequence to human proteins.

Prediction, Refinement, and Quality Assessment of the Tertiary Structure of the Developed Vaccine Construct

The designed vaccine was a reconstructed protein with no detectable homology (29). Phyre2 incorporates an ab initio folding simulation to model regions of proteins with no detectable homology. The Phyre 2 server (http://www.sbg.bio.ic.ac. uk/phyre2/) was used to predict the three-dimensional structure of the designed vaccine. The server generates a full-length 3D model of a protein sequence by employing both multiple template modeling and simplified ab initio folding simulation (29).

To enhance the overall and partial structural quality of the protein, the output 3D structure of the final vaccine from the Phyre 2 server was further refined by the GalaxyRefine server (http://galaxy.seoklab.org/cgi-bin/submit.cgi?type=REFINE) (30). The GalaxyRefine server predicted five refined models of our developed vaccine construct, in which Model 1 was made by the structural perturbation based simply on the clusters of the side chains; whereas, Models 2–5 were generated by deeper perturbations of loops and secondary structural elements (30).

For the assessment of the tertiary structure of the final vaccine protein, a Ramachandran plot was performed by the SWISS-MODEL workspace (https://swissmodel.expasy.org/assess) (31). The Ramachandran plot illuminates favored regions for backbone dihedral angles against amino acid residues in protein structure (31). The Structure Assessment page shows the most relevant scores provided by Molprobity and help we easily identify where residues of low quality lie in their model or structure (31). Then, ProSA-web (https://prosa.services.came.sbg.ac.at/prosa.php) was employed in the final vaccine protein structure validation. A positive *Z*-score commonly means an erroneous or erratic section found in the generated 3D protein model (68).

Molecular Docking of the SARS-CoV-2 Vaccine Construct With the Related Antigenic Recognition Receptor

To revealing the binding affinity between the vaccine construct and antigenic recognition receptors of toll-like receptor-3 (TLR3, 2A0Z) and major histocompatibility complex (MHC-I, 4WUU, and MHC-II, 3C5J) present on the surface of immune cells (69). Docking analysis was performed using the ClusPro server (https://cluspro.bu.edu/login.php?redir/queue.php). TLR3 act as receptors for antigenic recognition. The ClusPro server computed the models based on electrostatic interactions and desolvation energy (69). To reconfirm the binding affinity of the designed vaccine construct between these receptors, the PatchDock server (https://bioinfo3d.cs.tau.ac.il/PatchDock/) was used for docking (70). The server predicted the potential complex with the help of three algorithm-molecular shape representations, surface patch matching, filtering, and scoring (70). After the acquisition of the output from the PatchDock server, the complexes were refined by the FireDock algorithm, which predicted the optimal complex with the aid of energy functions (70).

Molecular Dynamic Simulation

The pdb file of vaccine protein and receptor complex (TLR3, MHC-I, and MHC-II) were used to start the molecular dynamic (MD) simulations. The complexes were placed in a octahedron box of water molecules represented by the three-point charge SPC model, whose boundary is at least 10 Å from any protein atoms. The solvated protein was subsequently neutralized by chloridions. Covalent bonds involving hydrogen atoms were constrained using the LINCS algorithm, and long-range electrostatic interactions were treated with particle-mesh Ewald employing a real-space cutoff of 10 Å. The system was first briefly minimized with backbone atoms restrained to the initial coordinates to remove close contacts, and the restrained system was gradually heated to 300 K under constant volume conditions in 100 ps. Each system was equilibrated for 1 ns using the constant isothermal-isobaric ensemble at 1 atm and 300 K without any restraints. The Parrinello-Rahman barostat and a V-rescale thermostat were used with an integration time step of 2 fs. Production run MD simulations were performed for 10 ns with coordinates recorded every 10 ps. All simulations were performed using GROMACS 2018.2 along with the GROMOS96 54a7 force field (16, 24).

Codon Adaptation and *in silico* Cloning

For the purpose of cloning, codon adaptation of the designed vaccine was performed for analyzing the codon usage by the prokaryotic organism (*Escherichia coli, E. coli*). The Java Codon Adaptation tool (http://www.jcat.de/) was used to optimize codon (71). Then the secondary structure of mRNA was predicted by Mfold (http://unafold.rna.albany.edu/?q=mfold) (72). For raising the expression efficiency of the final vaccine protein, the *E. coli* K12 strain was chosen. For the valid translation of the vaccine gene, we proofread and avoided rho-independent transcription termination, prokaryote ribosome binding site, and cleavage site of restriction enzymes. Restriction endonuclease sites XhoI and BamHI were appended to *N* and *C* terminals of vaccine, respectively. Then, it was inserted into the pET28a (+) vector between the XhoI and BamHI. The flow chart of the designed work is shown in **Figure 1**.

RESULTS

The strategy of vaccine construction is presented in **Figure 1**.

Antigenicity Analysis of SARS-CoV-2 and Selection of Protein Sequences for Vaccine Construction

The proteome of SARS-CoV-2 was retrieved, which comprised 27 proteins. The reference sequences of those proteins were retrieved in the FASTA format and their details are presented in **Table 1**. Five proteins with <100 amino acid sequences are too short to predict epitopes (ORF6 protein, ORF10 protein, ORF7b protein, nsp11, and envelope protein) were excluded.

In order to develop a subunit vaccine, it is critical to identify candidate proteins that are important for inducing a protective immune response (27). The remaining 22 proteins sequence were relayed to the VaxiJen server to determine their antigenicity based on the antigenic scores (**Table 1**). Proteins with antigenic scores >0.5 were selected for further analysis (28). Nine proteins, namely ORF7a protein, ORF8 protein, nsp9, nsp6, nsp3, endoRNAse, ORF3a protein, membrane glycoprotein, and nucleocapsid phosphoprotein were finally selected for further epitope prediction.

There is no available experimental structures of these nine proteins, we predicted homology models for the nine proteins applying the normal mode of phyre2 online server. The most suitable templates for the nine proteins were identified to be the PBD entries (**Table S1**). All of the modeled structures were showed over 90% residues in the Ramachandran favored region **Figure S1** and **Table S2**.

Identification of Cytotoxic T Cell Epitopes

The prediction of CTL epitopes (9 mer) was performed by the NetCTL server. The binder sites were determined based on

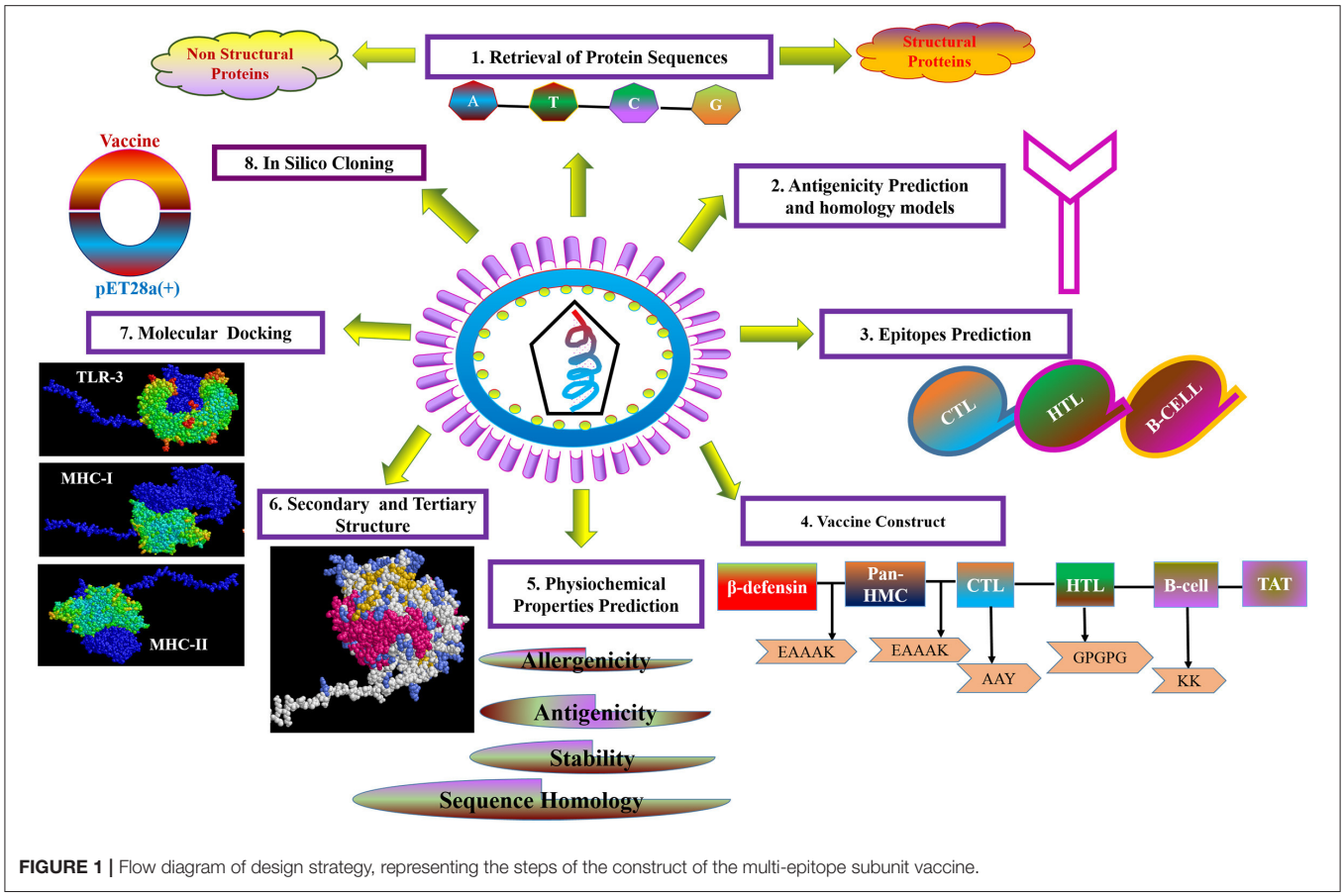


FIGURE 1 | Flow diagram of design strategy, representing the steps of the construct of the multi-epitope subunit vaccine.

three supertypes (A2, A3, and B7), with a 95% coverage rate of the world's population. Nine proteins were selected based on antigenicity. One epitope of each supertype was selected based on the highest score and an IC50 value < 500 nm. Then the specific Treg-inducing epitopes were excluded by Epitoolkit. A total of 18 epitopes were selected from nine proteins as the candidates for the construction of the vaccine (**Table 2**).

Identification of Helper T Lymphocyte Epitopes

The HTL epitopes (15 mer) were evaluated for three HLA supertypes: HLA-DR (DRB1*01:01, DRB1*07:01, DRB1*09:01, DRB3*01:01, DRB4*01:01); HLA-DQ (DQA1*01:01/DQB1*05:01, DQA1*01:02/DQB1*06:02, DQA1*03:01/DQB1*0:02, DQA1*04:01/DQB1*04:02, DQA1*05:01/DQB1*02:01, DQA1*05:01/DQB1*03:0 1); and HLA-DP (DPA1*01/DPB1*04:01, DPA1*01:03/DPB1*02:01, DPA1*02:01 /DPB1*01:01, DPA1*02:01/DPB1*05:01, DPA1*03:01/DPB1*04:02). We sorted the top epitopes with the lowest scores (low scores indicated the highest binding capability) from three supertypes. The best candidate was then selected based on positive IFN-γ induction and an IC50 < 500 nm. Then the specific Treg-inducing epitopes were excluded by Epitoolkit. Thus, a total of 14 epitopes were selected for vaccine design (**Table 3**).

Identification of Line and Conformational B-Cell Epitopes

We used the ABCpred and BepiPred servers to identify the line B cell candidate epitopes. All predicted epitopes from both servers were compared, and only the overlapping epitopes were selected for the development of the vaccine. The line epitopes identified by ABCpred had prediction scores ranging from 0.52 to 0.93, and line epitopes identified by BepiPred had prediction scores ranging from 0.5 to 1. Among these line epitopes, only 12 (16 mer) were found to be common or partly common in both servers (**Table 4**). These 12 line epitopes were selected for vaccine construction (**Table 4**).

The non-continuous B cell epitopes were predicted by the ElliPro severs, a total number of 27 non-continuous B cell epitopes were generated from ElliPro. Amino acid residues, sequence location, the number of residues, and the PI scores of the predicted conformational epitopes are shown in **Table 5** and the graphical depiction of these epitopes can be seen in **Figure S2**. Twenty-four epitopes were excluded because it added the allergenicity of vaccine, three epitopes were marked red and selected for vaccine construction.

Construction of the Subunit Vaccine

The best candidate epitopes were used for the construction of the vaccine. A total of 18 CTL epitopes, 14 HTL epitopes, 12

TABLE 2 | Predicted cytotoxic T lymphocyte (CTL) epitopes of SARS-CoV-2 proteins utilized for the construction of a multi-epitope subunit vaccine.

Protein	CTL epitopes predicted using the NetCTL server		
	A2 supertype (IC50)	**A3 supertype (IC50)**	**B7 Supertype (IC50)**
ORF7 protein	KLFIRQEEV (58.81)	TLCFTLKRK (219.46)	SPIFLIVAA (231.29)
nsp9	ALLSDLQDL (8.28)		
nsp6	FLLPSLATV (2.7)	SAFAMMFVK (92.76)	MPASWVMRI (171.46)
nsp3		VMYMGTLSY (72.50)	
endoRNAse	LLLDDFVEI (21.12)		SPFGHSLTL (10.75)
ORF3a protein		IMRLWLCWK (98.03)	IPIQASLPF (13.56)
Membrane glycoprotein	GLMWLSYFI (11.32)	LSYFIASFR (138.74)	LPKEITVAT (244.01)
Nucleocapsid phosphoprotein	LLLDRLNQL (84.26)	KTFPPTEPK (69.08)	FPRGQGVPI (3.82)

The half-maximal inhibitory concentration (IC50) value was > 500 nm, which ensured a higher binding capability of the selected epitopes to MHC molecules.

TABLE 3 | Predicted Helper T lymphocyte (HTL) epitopes of SARS-CoV-2 proteins utilized for the construction of a multi-epitope subunit vaccine.

Epitope	Allele (score)	IC50
ORF8 protein		
HFYSKWYIRVGARKS	HLA-DRB1*07:01 (0.06)	32.2
	HLA-DRB1*01:01 (0.07)	39.5
DFLEYHDVRVVLDFI	HLA-DQA1*05:01/DQB1*02:01 (0.01)	276
	HLA-DQA1*01:01/DQB1*05:01 (0.5)	101.9
IHFYSKWYIRVGARK	HLA-DPA1*02:01/DPB1*01:01 (0.01)	281.8
	HLA-DPA1*03:01/DPB1*04:02 (0.02)	42.8
	HLA-DPA1*01:03/DPB1*02:01 (0.09)	79.4
nsp9		
KGLNNLNRGMVLGSL	HLA-DQA1*05:01/DQB1*03:01 (0.04)	62
	HLA-DQA1*01:02/DQB1*06:02 (0.62)	95
GPKVKYLYFIKGLNN	HLA-DPA1*01:03/DPB1*02:01 (0.01)	61
	HLA-DPA1*02:01/DPB1*05:01 (0.02)	79.7
	HLA-DPA1*02:01/DPB1*01:01 (0.52)	109.3
nsp3		
TAFGLVAEWFLAYIL	HLA-DQA1*05:01/DQB1*02:01 (0.01)	50
	HLA-DQA1*01:01/DQB1*05:01 (0.14)	46.4
AAIMQLFFSYFAVHF	HLA-DPA1*01:03/DPB1*02:01 (0.01)	8.7
	HLA-DPA1*01/DPB1*04:01 (0.01)	78.5
	HLA-DPA1*02:01/DPB1*01:01 (0.02)	110.8
endoRNAse		
MEIDFLELAMDEFIE	HLA-DQA1*03:01/DQB1*03:02 (0.01)	97.6
	HLA-DQA1*05:01/DQB1*02:01 (0.03)	12.9
	HLA-DQA1*01:01/DQB1*05:01 (0.07)	37.9
GLAKRFKESPFELED	HLA-DPA1*01/DPB1*04:01 (0.02)	108.6
	HLA-DPA1*02:01/DPB1*05:01 (0.12)	453
ORF3a		
ACFVLAAVYRINWIT	HLA-DRB1*07:01 (0.01)	19.9
	HLA-DRB1*01:01 (0.02)	12.4
membrane glycoprotein		
ACFVLAAVYRINWIT	HLA-DQA1*05:01/DQB1*02:01	321.4
KLIFLWLLWPVTLAC	HLA-DPA1*03:01/DPB1*04:02 (0.01)	187.6
	HLA-DPA1*02:01/DPB1*01:01 (0.52)	133.8
	HLA-DPA1*01:03/DPB1*02:01 (0.75)	21.3
DDQIGYYRRATRRIR	HLA-DRB1*01:01 (0.01)	223
	HLA-DRB1*07:01 (0.01)	43
	HLA-DRB3*01:01 (0.01)	79.2
nucleocapsid phosphoprotein		
GKMKDLSPRWYFYYL	HLA-DPA1*01:03/DPB1*02:01 (0.08)	194.7

The half-maximal inhibitory concentration (IC50) value was < 500 nm, which ensured a higher binding capability of selected epitopes to MHC molecules.

linear, and three non-continuous B cell epitopes were fused together with the aid of linker sequences. The CTL epitopes were linked by AYY (The AAY liner helps the epitopes produce suitable sites for binding to TAP transporter and enhances epitope presentation), the HTL epitopes were combined with the aid of GPGPG (The GPGPG linker stimulate HTL responses and conserve conformational dependent immunogenicity of helpers as well as antibody epitopes), and B cell epitopes were merged with the aid of KK. The final to enhance vaccine immunogenicity, the human β-defensin-3 sequence (45aa) and pan-HLA DR binding epitopes (The pan-HLA DR binding epitopes in vaccine construct facilitating binding to many different types of mouse and human MHC-II alleles to induce CD4-helper cell responses.) was added to the N-terminal of the vaccine with the aid of the EAAK linker. To enable the intracellular delivery of the modeled vaccine, a TAT sequence (11aa) was appended to C-terminal. The vaccine was developed to be 864 amino acids in length (**Figure S3**). The sequence homology of final vaccine protein to human protein sequence shown that there were no significant alignments (**Figure S4**).

Evaluation of Allergenicity, Antigenicity, and Physiochemical Parameters of the Vaccine

The allergenic character of the vaccine was determined by the AlgPred server and was based on the hybrid approach (SVMc + IgE epitope + ARPs BLAST + MAST) with a 93.5% coverage. The vaccine was non-allergen with 84% accuracy and 82.78% sensitivity at threshold value was −0.2. Similarly, the antigenic nature of the vaccine construct was evaluated and showed that the protein was a favorable antigen with a global prediction score of a protective antigen of 0.5308 (Probable antigen). The default threshold value for antigenicity was 0.4 in the virus model.

Moreover, the vaccine constructs contained 864 amino acids, and its molecular weight was 95.4 kDa. The theoretical pI was predicted to be 9.71. The vaccine contained 63 negatively charged residues and 125 positively charged residues. The vaccine construct was composed of 13,541 atoms, and its chemical

TABLE 4 | Predicted line B cell (BCL) epitopes of SARS-CoV-2 proteins utilized for construction of a multi-epitope subunit vaccine.

Protein	Sequence	Start position	Score
ORF7 protein	SGTYEGNSPFHPLADN	37	0.92
ORF8 protein	KSPIQYIDIGNYTVSC	68	0.88
	HFYSKWYIRVGARKSA	40	0.87
nsp9	KGPKVKYLYFIKGLNN	81	0.93
	AGTTQTACTDDNALAY	16	0.91
endoRNAse	DFLELAMDEFIERYKL	212	0.76
ORF3a protein	TSPISEHDYQIGGYTE	176	0.93
Membrane	HVQIHTIDGSSGVVNP	243	0.91
glycoprotein	YRIGNYKLNTDHSSS	199	0.69
	NGTITVEELKKLLE	5	0.61
Nucleocapsid phosphoprotein	KSAAEASKKPRQKRTA	249	0.93
	EGALNTPKDHIGTRNP	136	0.93

formula was $C_{4395}H_{6791}N_{1153}O_{1174}S_{28}$. The computed instability index was 32.84, which was <40, classifying the vaccine as a stable protein. The estimated half-life was 1 h *in vitro*. *In vivo*, the estimated half-lives in yeast and *Escherichia coli* are greater 30 min and 10 h, respectively. The aliphatic index of the vaccine construct was 79.29, which suggests a high thermostability. The GRAVY value of the vaccine construct was −0.215, which indicated the hydrophobicity of the protein.

The Immune Response Profile *in silico* Immune Simulation

The immune stimulation of the final vaccine was performed using C-ImmSim online server, which gives the immune profiles of the designed vaccine. The proliferation in the secondary and tertian immune response were identified by IgG1 + IgG2 and IgM, as well as, the decreasing of the antigen count IgG + IgM showed the proliferated (**Figure 2A**). The stimulation result revealed the development of immune response after immunization. B cell population was highly stimulated upon immunization (**Figure 2B**). Similarly, the cytotoxic and helper T cell levels were proliferated that suggested the development of secondary and tertian immune response (**Figures 2C,D**). During the exposure time, it was also observed that the production of IFN-γafter immunization (**Figure 2E**). These results were significant for the immune response against SARS-CoV-2. Hence,

Prediction, Refinement, and Quality Assessment of the Tertiary Structure of the Developed Vaccine Construct

The tertiary structure of the full-length vaccine sequence was predicted by Phyre 2, and it was applied for refinement and further analysis. Twenty-five templates were employing modeling as **Figure S5** shown. There were three templates from human defensin which were we added in to enhance the immunogenicity, others from virus (**Figure S5**). The immune epitopes were not structural homology to human proteins that could avoid inducing autoimmune. The secondary structure of the predicted model contained 18% alpha-helix, 21%TM helices 44% beta-sheets, and 27% disordered **Figure S6**.

To optimize the 3D structure of the modeled protein, the initial model was refined in the GalaxyRefine server. The GalaxyRefine server-generated five models based on the root-mean-square deviation (RMSD) and MolProbity algorithm. The details of the five models are shown in **Table S3**. Model 1 with the top Ramachandran favored, therefore selected for docking purposes (**Figure 3**). A model with more residues in the Ramachandran favored region, less in outliers region and rotamer region was considered as a more ideal one. The initial model generated from Phyre 2 server and refine model from GalaxyRefine were evaluated with the aid of the SWISS-MODEL workspace. The initial model was 63.46% of residues in the Ramachandran favored region, 19.49% in the Ramachandran outliers region, and only 10.22% in the rotamer region (**Figure 4**). The refine model was 89.1% of residues in the Ramachandran favored region, 2.09% in the Ramachandran outliers region, and only 0.15% in the rotamer region (**Figure 3**). Other favorable parameters of the refined model were as follows: GDT score of 0.9922, RMSD value of 0.260, MolProbability of 2.049, clash score of 8.9, and poor rotamers totaling 0.3 (**Table S1**).

The quality and potential errors in the final vaccine 3D model were verified by ProSA-web. The *Z*-score indicates overall model quality, the model with a lower *Z*-score was considered as the higher quality one. The *z*-score of the initial model was −2.81, refine model is −3.64 (**Figure 5**).

Molecular Docking of Final Vaccine Construct With the Relatively Antigenic Receptor

To further evaluate the binding affinity between the developed vaccine construct and the relative antigenic receptors (TLR3, MHC-I, and MHC-II), molecular docking was performed. The server yielded 44 candidate models with different binding energies. Twenty-nine model complexes of TLR3 and COVID-19 vaccine were determined, from which just one complex with the lower binding energy score of −1156.2 was selected to show (**Table 6** and **Figure 6**). A total of 29 model complexes of MHC-I and the COVID-19 vaccine were discovered, and the lowest binding energy score was −1346.8 (**Table 6** and **Figure 6**). A total of 29 complex models of MHC-II and the COVID-19 vaccine were predicted, among which, one model complex with the lowest binding energy score of −1309.1 was chosen to show (**Table 6** and **Figure 6**). Further, the vaccine construct was evaluated using the PatchDock server, which identified different models and produced a score table. The top 10 complexes identified were refined by the FireDock algorithm. Among those top 10 models, the model with the lowest binding energy was further selected to show in this paper. The refinement outcomes of TLR3 and the vaccine complex was solution number 1 with global energy of −38.40, attractive van der Waals energy (VdW) of −26.02, repulsive (VdW) of 8.62, and atomic contact energy of −11.06 (**Table 6** and **Figure 6**). The complex of MHC-I and the vaccine was ranked number nine, with global energy of −22.97, attractive VdW of −26.84, repulsive VdW of 12.82, and atomic

TABLE 5 | Predicted conformational B cell (BCL) epitopes of SARS-CoV-2 proteins.

R	Residues	NO.	score
ORF7a protein			
1	R25, G26, T27, T28, L30, K32, E33, P34, C35, S36, S37, G38, P45, H47, P48, L49, A50, D51, N52, K53, C58, C67, P68, D69, G70, V71, R80, S81, V82, S83, P84, K85, L86, F87, I88, R89, E91, E92, E95, L96	40	0.675
ORF8 protein			
2	C37, P38, I39	3	0.558
3	Q23, S24, C25, T26, Q27, H28, Q29, P30	8	0.556
nsp9			
4	K58, S59, D60, G61, T62, G63, T64	7	0.831
5	D47, V76, D78, T79, P80, K81, G82, P83, K84, V85, G104, A107, A108, T109, V110, R111,	17	0.729
6	N1, N2, E3, L4, S5, P6, V7, A8, L9, T34, T35, K36, G37, G38, E70, K92, G93, L94, N95, N96, L97	21	0.659
7	T18, T19, Q20, T21, A22, C23, T24, D25, L48, Q49, D50, L51	12	0.647
nsp6			
8	G258, L259, L260, P261	4	0.786
9	L275, L276, G277, V278, G279, G280, K281, P282, C283, I284	10	0.641
nsp3			
10	S675, S676, K677, T678, P679, E680, E681, H682, F683, I684, E685, T686, I687, S688, L689, A690, G691, S692, Y693, K694, D695, W696, S697, Y698, S699, G700, Q701, S702, T703, Q704, L705, G706, I707, E708, F709, L710, K711, R712, G713, D714, K715, S716, V717, Y718, Y719, T720, S721, N722, P723, T724, T725, F726, H727, L728, D729, G730, E731, V732, I733, T734, F735, D736, N737, L738, L741, R745	66	0.818
11	N922, L923, D924, S925, C926, K927, R928, V929, L930, N931, V932, V933, C934, K935, T936, C937, G938, Q939, Q940, Q941, T942, T943, L944, K945, G946, K962, K963, G964, V965, Q966, I967, P968, C969,T970, C971, G972, K973, Q974, A975, T976, K977, Y978, L979, V980, Q981, Q982, E983, S984, P985, F986	50	0.719
12	K839, P841, Q842, V843, N844, G845, L846, T847, W851, A852, D853, N854,N855, C856, L956, S957, A991, P992, P993, A994, Q995, Y996, E997, L998, K999, H1000, G1001, T1002, F1003, T1004, E1008, Y1009, T1010, G1011, N1012, Y1013, Q1014, C1015, G1016, H1017, K1019, T1022, S1023, K1024, E1025, T1026, L1027, Y1028, C1029, I1030, D1031, G1032, A1033, L1034, L1035, T1036, K1037, S1038, S1039, E1040, Y1041, K1042, G1043, P1044, I1045	65	0.648
13	D806, D807, T808, L809, V811, E812, F814	7	0.62
14	K1051, E1052, N1053	3	0.602
endoRNAse			
15	S1, L2, E3, N4, V5, A6, F7, N8, V9, V10, N11, K12, G13, H14, F15, D16, G17, Q18, Q19, G20, E21, V22, P23, V24, S25, I26, I27, N28, N29, T30, V31, Y32, T33, K34, V35, D36, G37, V38, D39, V40, E41, L42, E44, N45, K46, T47, T48, L49, P50, V51, N52	51	0.755
16	E145, G146, S147, V148, K149, G150, L151, G169, E170, A171, V172, K173	12	0.707
17	L200, P205, S207, M209, I211, D212, L214, E215, L216, A217, M218, D219, E220, F221, I222, E223, R224, Y225, L227, E228, G229, Y230, A231, F232, E233, H234, I235, Y237, G238, D239, F240, S241, H242, S243, Q244, L245, G246, K256, R257, F258, K259, E260, S261, P262, E264, F279, T281, D282, A283, Q284, T285, G286, S287, S288, K289, C290, K307, S308, Q309, D310, L311, S312, V313, V314, S315, K316, V317, M330, L331, W332, C333, K334, D335, G336, H337, V338, E339	77	0.699
18	T98, I99, G100, C102, S103, M104, T105, D106, I107, A108, K109, K110, P111, T112, E113, T114, I115, C116, A117, P118, L119, T120, G125, R126, V127, D128, G129, V131, D132, L133, F134, R135, N136, A137, R138, N139, K181, V182, D183, G184, V185, V186, Q187	45	0.655
19	Q152, P153, S154	3	0.581
ORF3a			
20	H78, C81, N82, L83, L84, L85, L86, F87	8	0.69
21	V97, A98, A99, G100, L101, E102, F105, Y109	8	0.668
22	Q70, L71, K75	3	0.613
Membrane glycoprotein			
23	N21, L22, V23, I24	4	0.731
24	N5, G6, T7, I8, T9, V10, E11, K	8	0.588
Nucleocapsid phosphoprotein			
25	K338, L339, D340, D341, K342, D343, P344, N345, F346, K347, D348, V350, I351, N354, I357	15	0.747
26	G316, M317, S318, R319, I320, G321, M322, E323, V324, T325, P326, S327, G328, T329, W330, L331, G335	17	0.689
27	A252, E253, A254, S255, K256, K257, P258, K261, R262, A264, T265, K266, A267, Y268, N269, Q272, G278, P279, E280, T282, Q283, N285, G287, D288, Q289, E290, R293, Q294, D297, Y298, K299, H300, D358, A359, Y360, K361, T362, F363, P364	36	0.567

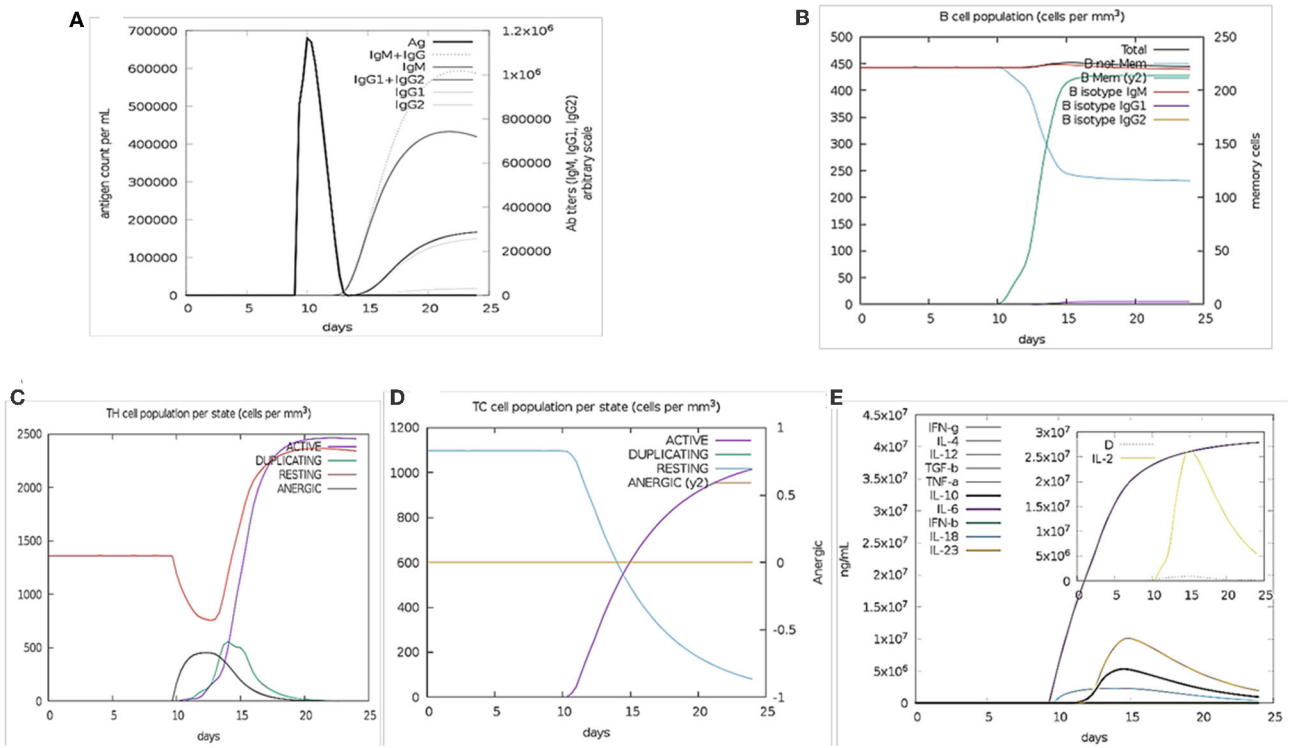


FIGURE 2 | Immune Simulation results by C-ImmSim. **(A)** The immunoglobulins production represent proliferation of immune response after the vaccine administration. Various subtypes of immunoglobulin are represented as colored peaks. **(B)** The active B-cell population is observed with the administration of vaccine. **(C)** The generation of Helper-T cells. **(D)** The generation of cytotoxic-T cells were found after the vaccine injection. The RESTING indicates to the cells, which were not shown to the antigens while ANERGIC indicates the tolerance level of antigen. **(E)** The cytokine profile shows that the induced IFN-γlevel upon administration of vaccine. The inset graph indicating the Simpson Index, D of IL- 2. Simpson Index, D was inferred as the measurement of diversity.

contact energy of −1.79 (**Table 6** and **Figure 6**). The complex of MHC-II and the vaccine was ranked number three, with global energy of −27.52, attractive VdW of −26.86, repulsive VdW of 10.93, and atomic contact energy of 0.77 (**Table 6** and **Figure 6**).

Molecular Dynamic Simulation

To accomplish the estimate of the stability of the vaccine-receptor complex, we performed the simulation of the docked complexes (vaccine and TLR-3, MHC-I, and MHC-II) with the help of GROMACS. Then, various analysis like energy minimization, pressure assessment, temperature, and potential energy calculations were performed. The temperature and pressure of the simulation system during the production run was around 300 K and 1 atmosphere, respectively, indicating a stable system and successful md run. The temperature and pressure of the three simulation systems (vaccine and TLR-3, MHC-I, and MHC-II complexes) during the production run were around 300 K and 1 atmosphere, respectively, indicating the stable systems and successful MD run (**Figures 7A–F**). The complex root mean square deviation (RMSD) plot represents the structural fluctuation of the overall structure of the complex of vaccine and immune receptor. The RMSD of vaccine-TLR3 complex has large fluctuation during 0–6 ns simulation. After 6 ns, the RMSD value was kept around 1.25 nm, indicating that the conformation of this complex was stable (**Figure 7G**). Otherwise, the RMSD of vaccine-MHC-I and -MHC-II complexes has large

FIGURE 3 | Refinement of the SARS-CoV-2 vaccine construct. Representative 3D image of the tertiary structure of the 2019nCOV vaccine after modeling.

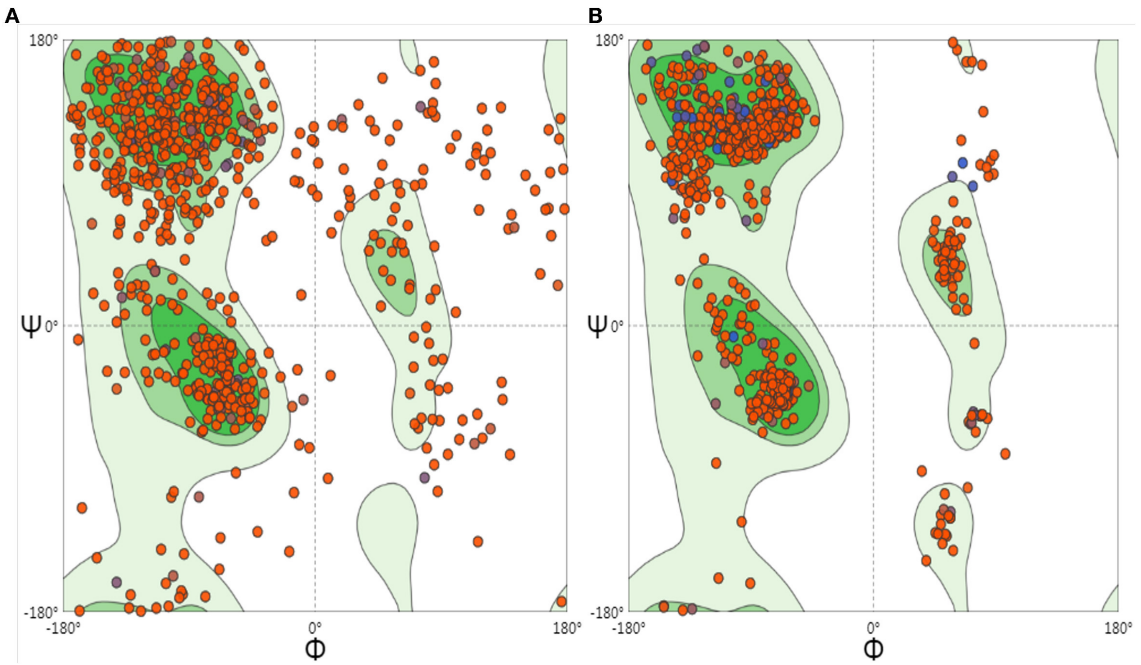


FIGURE 4 | Ramachandran plots to initiate and refine the 3D structure of the vaccine construct illustrated using the SWISS-MODEL/Structure Assessment. **(A)** Shows the Ramachandran plot of initiate model, **(B)** shows the Ramachandran plot of refining the model.

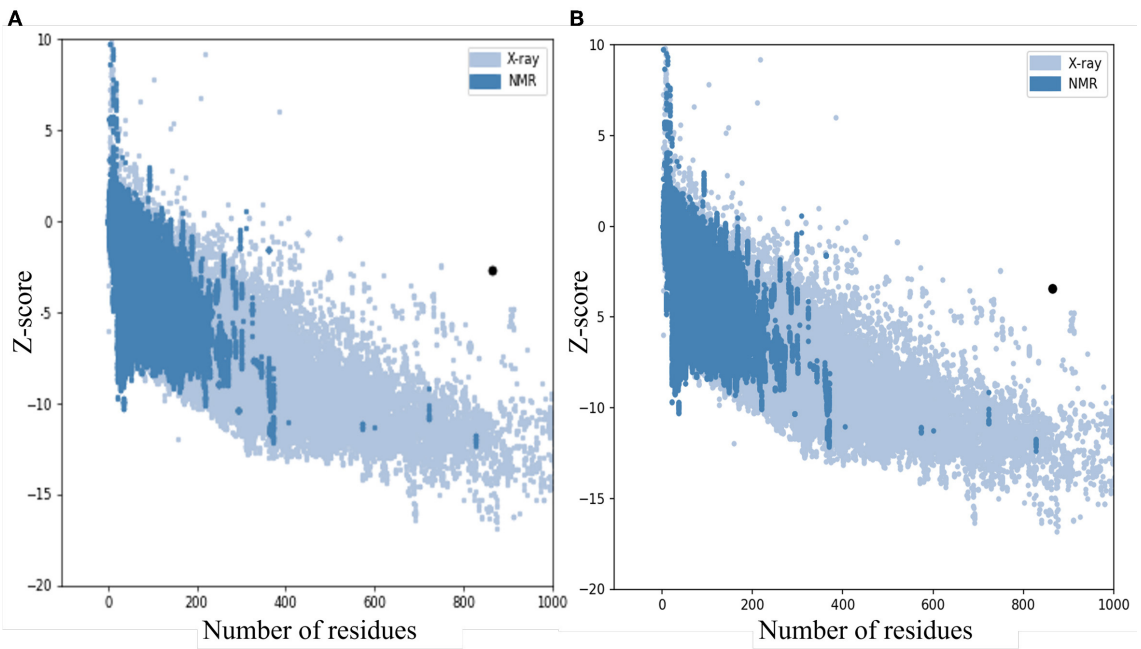


FIGURE 5 | *Z*-Score plot for the 3D structure of the final vaccine. The *Z*-score of **(A)** the initial model is 2.81 and **(B)** The *z*-score of the refined model is 3.64, both of two models not in the range of native protein conformation. *Z*-Score plot contains *z*-scores of all experimental protein chains in PDB determined by NMR spectroscopy (dark blue) and X-ray crystallography (light blue).

fluctuation during 0–4 ns simulation. After 4 ns, the RMSD value were kept around 1 nm, indicating that the conformation of the two complexes were stable (**Figures 7H,I**). Next, the root medium square fluctuation (RMSF) indicates the flexibility of the residue in the docking complex. From the results of vaccine-TLR3, MHC-I, and MHC-II complexes, residue 200–600

TABLE 6 | Molecular docking of final vaccine constructs with TLR3, Mda5, and MHC-II.

Receptor	ClusPro		FireDock			
	Center	Lowest energy	Glob[a]	aVdW[b]	rVdW[c]	ACE[d]
TLR3	−1156.2	−1416.4	−38.40	−26.02	8.62	−11.06
MHC-I	−1346.8	−1379.8	−22.97	−26.84	12.82	−1.79
MHC-II	−1309.1	−1389.3	−27.52	−26.86	10.93	0.77

[a]*Glob, Global Energy.*
[b]*aVdW, attractive van der Waals energy.*
[c]*rVdW, repulsive van der Waals energy.*
[d]*ACE, atomic contact energy.*

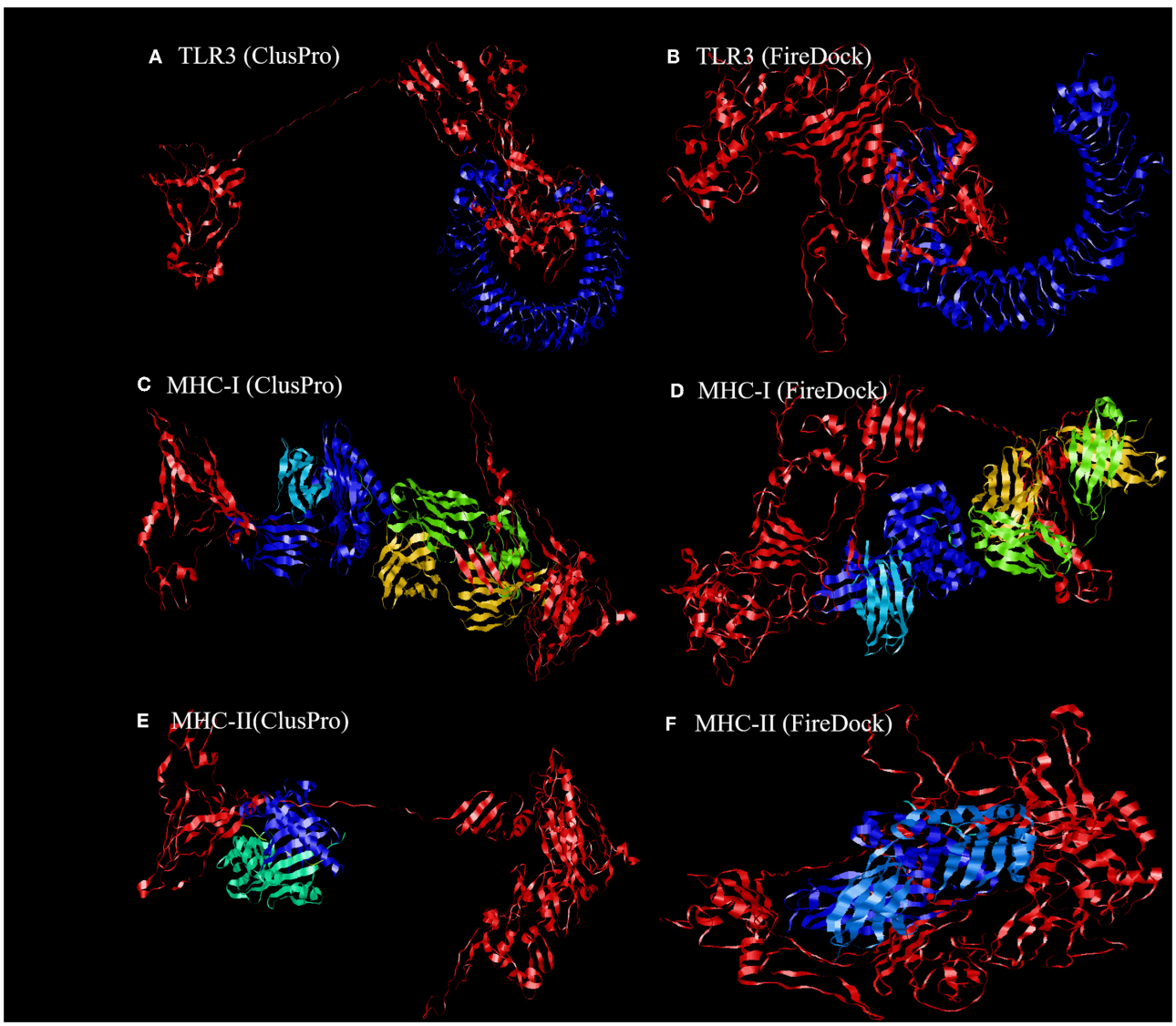


FIGURE 6 | Representation of the ligand-receptor docked complex. **(A,C,E)** show the molecular docking of the vaccine construct (red color) and TLR-3, MHC-I, and MHC-II receptors (other colors) illustrated using the ClusPro software. **(B,D,F)** show the molecular docking of the vaccine construct (red color) and TLR-3, MHC-I, and MHC-II receptors (other colors) illustrated using PatchDock to verify the stability of the docked complex.

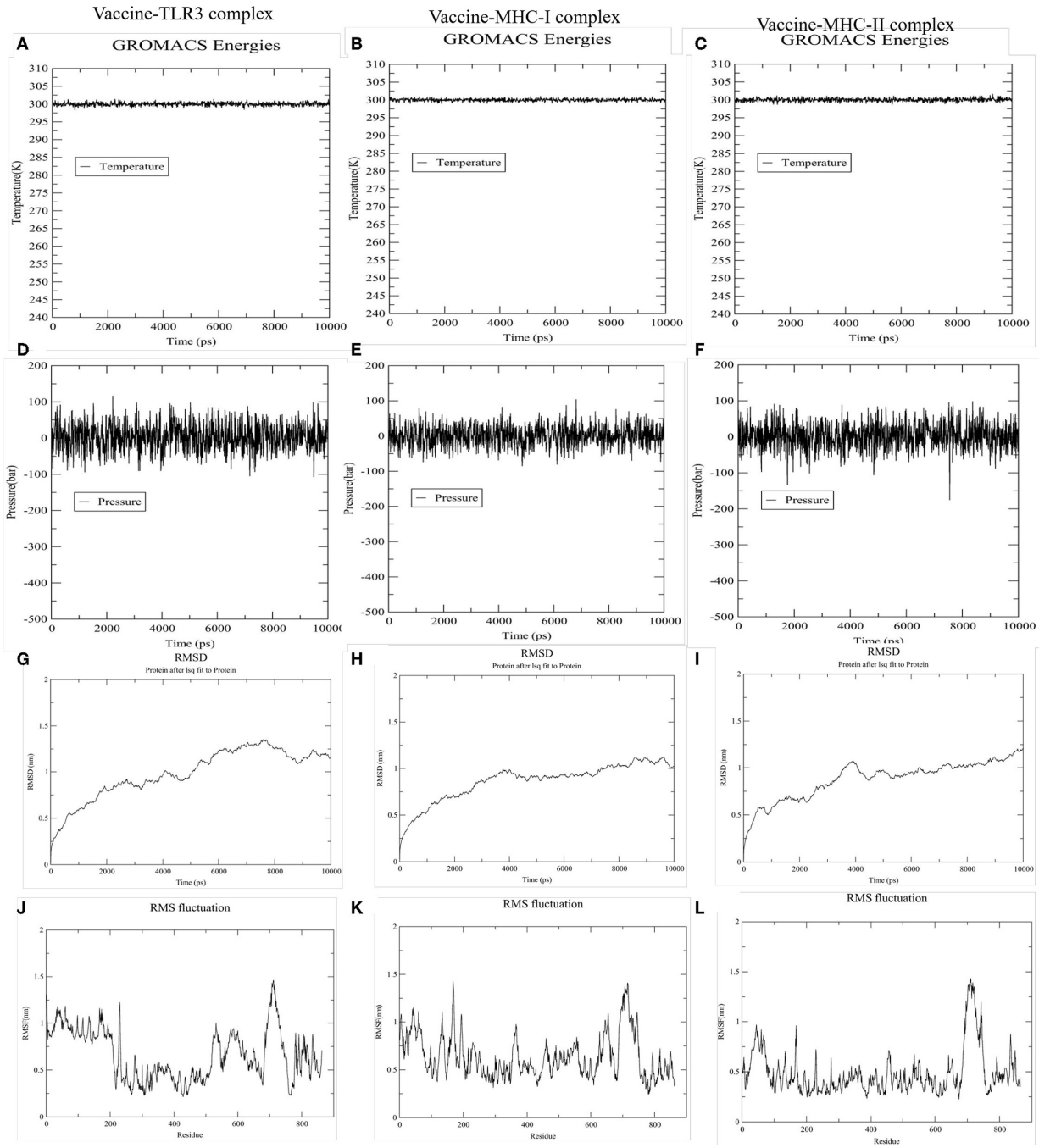


FIGURE 7 | The results of molecular dynamics simulation of vaccine and immune receptors. **(A–C)** show the equilibration phase ensembles-temperature (constant at 300 k for 100 ps) of the complex of vaccine-TLR3, MHC-I, and MHC-II, respectively. **(D–F)** represent the pressure (displaying fluctuations at 1 bar value for 100 ps) of the complex of vaccine-TLR3, MHC-I, and MHC-II, respectively. **(G–I)** suggest the RMSD (root mean square deviation) plots reflect the stability between the vaccine and TLR-3, MHC-I, and MHC-II receptor, separately. Whereas, **(J–L)** RMSF (root mean square fluctuation) reflect the flexibility and fluctuation of the amino-acids residues in the side chain of docked complexes (the complex of vaccine-TLR3, MHC-I, and MHC-II), separately.

has low RMSF value, indicating these residues has low structural flexibility. By contrast, residue 0–200 and 600–800 has relatively higher RMSF value, indicating the larger flexibility during those regions (**Figures 7J–L**).

In silico Cloning and Prediction of RNA Secondary Structure

To fuse the final vaccine to an expression vector, codon conversion of the vaccine protein was performed by the Java

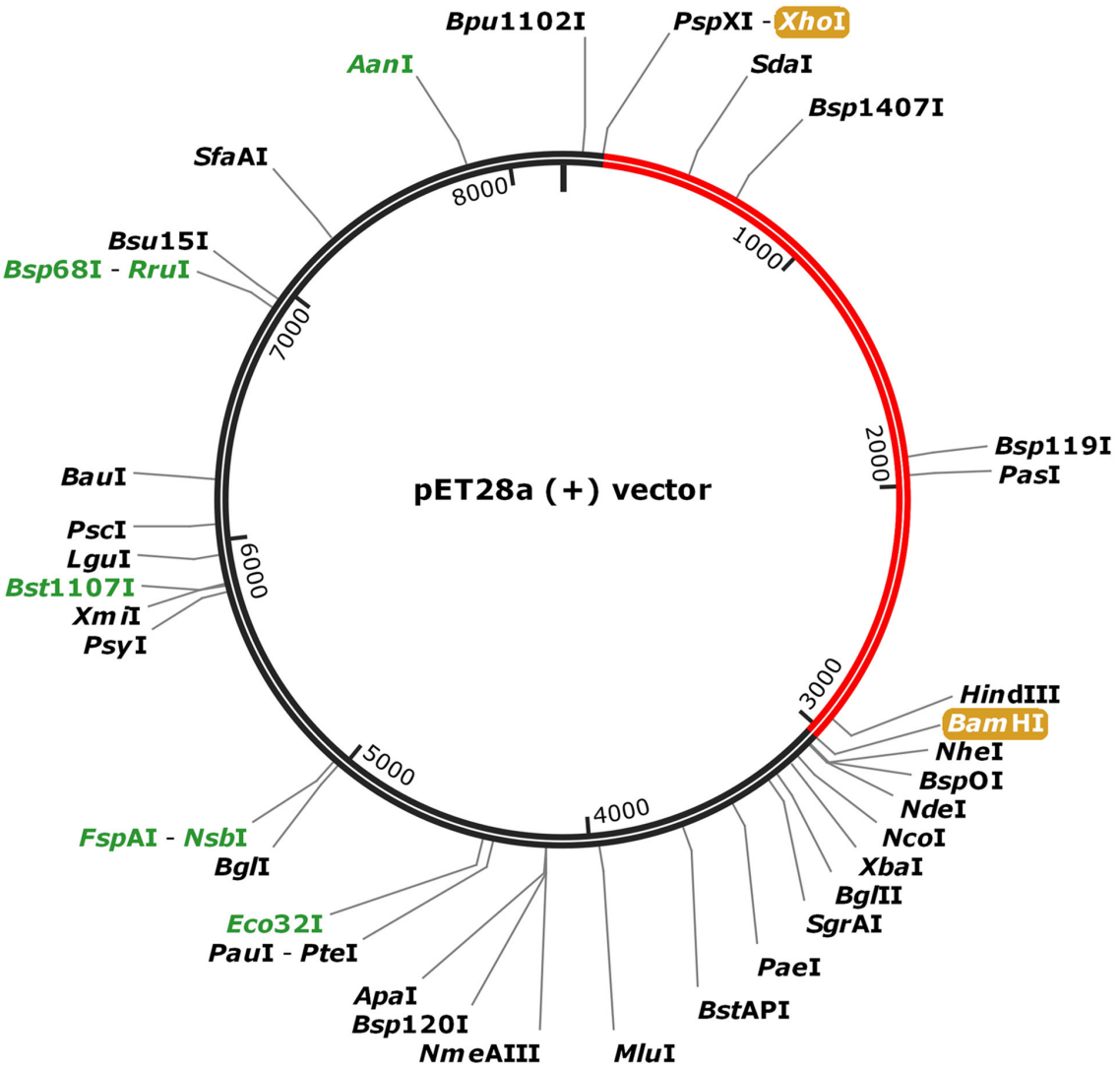


FIGURE 8 | *In silico* cloning of the SARS-CoV-2 vaccine in the vector, pET28a (+). Red areas represent the COVID-19 vaccine, while the black areas represent the expression vector, pET28a (+).

Codon Adaptation tool. Restriction site XhoI and Bam HI were added to *N* and *C* terminals of the codon sequence, then was inserted into the pET28a (+) vector between the XhoI and BamHI (**Figure 8**). The RNA secondary structure using the Mfold program was generated foldings contain 4,381 base pairs out of 2.3% in the energy dot plot. Mfold predicted an identical secondary structure of 4,381 bp formed by nucleotide fragments (**Figure S7**).

DISCUSSION

SARS-CoV-2 is characterized by high infectivity and high transmission speed; thus, a prophylactic vaccine is needed (11). The availability and advantages of the multi-peptide vaccine developed by immunoinformatics methods have been confirmed by previous studies (73, 74). Ojha et al. used the immunoinformatics methods to develop a multiepitope subunit vaccine to Epstein-Barr virus-associated malignancy (73). In recent studies, genomics and proteomics information of SARS-CoV-2 have been retrieved, stored, and utilized (75, 76). In the present research, we tried to develop a multi-epitope subunit prophylactic vaccine of SARS-CoV-2, with the help of immunoinformatics tools.

A line of research have tried to develop the vaccine of SARS-CoV-2 by immunoinformatics tools. Baruah and Bose (15) used immunoinformatics tools to discover cytotoxic T lymphocyte (CTL) and B cell epitopes for the spike protein of SARS-CoV-2. Then, Abraham et al. developed a multi-epitope vaccine that was designed using immunoinformatics tools that potentially trigger

both CD4+ and CD8+ T-cell immune responses (16). Most of those research just focus on the spike protein-based vaccine. A vaccine based on the spike protein could induce antibodies to block SARS-COV-2 binding and fusion or neutralize virus infection (18), as well as induce harmful immune responses that cause liver damage (19). Other proteins should be ideal candidates for designing vaccines.

In the present report, we selected nine proteins with positive antigenicity for further epitope prediction. All proteins from SARS-CoV-2 with <100 amino acid sequences were excluded, and the antigenic nature of the remaining proteins was evaluated. This method can facilitate the discovery of potential antigens of SARS-CoV-2 when the precise immunity mechanisms are unknown. To design an effective vaccine, we selected the SARS-CoV-2 protein through the above-mentioned methods for epitope prediction. In recently, Asaf et al. reported that identify multiple epitopes for CD4 $^{+}$ 12 and CD8 $^{+}$ T cells based on muti-protein (77). Their protein list was the same as this in our research. In Asaf's report, they just predicted the T cell epitopes, non-B cell, B cell peptide was not predicted (77).

The B cell epitopes are antigenic determinants from the antigen that are recognized by the B cell surface membrane receptor and evoke the production of specific antibodies. The persistent challenge in immunological prediction tools is the prediction of epitopes to a higher level of accuracy (78). To determine accurate linear B cell epitopes from the antigenic proteins, we used two bioinformatics tools based on different algorithms of prediction. We identified nine overlapping linear B cell epitope candidates from two different bioinformatics tools. This method was superior to the prediction of epitopes from a single tool (78). Moreover, we also have predicted the non-continue B-cell epitopes.

The B cell immune response is preferred in the design of a vaccine. However, T cells may also elicit a strong immunoreaction. The vaccine that activates both CTLs and HTLs should be more effective than a vaccine that only targets CTL responses (79). To generate a more effective vaccine, we predicted both CTL epitopes and HTL epitopes. The T cell epitopes were decomposed fragments from the antigen presented by the MHC molecules of T cells and stimulated the production of effector T cells, immunological memory T cells, and IFN-γ. The cell-mediated immune response induced by CTLs plays a vital role in the defense against viral infections through the recognition of intracellular viral pathogens by MHC class I molecules.

In the present report, MHC-I binding epitopes were predicted by choosing A2, A3, and B7 alleles, which cover ∼95% of world's population. We selected 18 CTL epitopes. The HTLs play a vital role in the antiviral immune response by producing IFN-γ. Moreover, HTLs are able to induce and maintain CTL responses. Furthermore, 14 HTLs epitopes were chosen based on both the binding capability and IFN-γ induction. Bhattacharya et al. also used the spike protein sequence predicted for MHC-I and MHC-II epitopes of SARS-CoV-2, but not predicted capability of producing IFN-γ (80). The T cell epitopes enhanced IFN-γ inducing capability, which evokes both the native and specific immune responses by activating macrophages and natural killer cells, and augmenting the response of the MHC to the antigen (81, 82).

In this study, the immunogenic epitopes from B cells, CTLs, and HTLs were chosen to develop a more valid, reliable, and effective vaccine against SARS-CoV-2. A multiepitope approach was used by splicing together epitopes with the aid of their respective linkers. To improve the immunogenicity of this multiepitope vaccine, an adjuvant β-defensin and pan-HLA DR binding epitopes (13aa) were fused to the N-terminal with the aid of an EAAAK linker, then A TAT sequence (11aa) was appended to C-terminal with the added of KK. The final vaccine constituted 864 amino acids. The allergenicity, antigenicity, and stability of the designed vaccine constructs were then evaluated. The tertiary structure of the generated vaccine was predicted by using the Phyre 2 server and then refined by the GalaxyRefine server. The binding affinity of complexes of the developed vaccine and receptors, in which TLR-3, MHC-I, and MHC-II (present on the surface of the immune cell) were confirmed by the ClusPro server was based on molecular docking.

Furthermore, to ensure the translation efficiency of the designed vaccine in a specific expression system, the mRNA of the vaccine was enhanced with the aid of the Java Codon Adaptation Tool. The restriction enzyme cutting sites of Xho? and BamH? were then appended to the *N* and *C* terminals, respectively. The vaccine sequence was subsequently cloned in pET28a (+), the expression vector. Further experimental validation of the safety and efficacy of the designed vaccine for SARS-CoV-2 is warranted.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article.

AUTHOR CONTRIBUTIONS

RD and ZC performed the experiments. RD and YZ wrote the paper. YZ and FY edited the final version. All authors participated in the experimental design, data analysis, and agreed with the final version of the paper.

FUNDING

This work was partly supported by grants from the Special Fund for Basic Scientific Research Operating of Central Public Welfare Research Institutes, the Chinese Academy of Medical Sciences (2019PT320003), and Guizhou High-Level Innovative Talents Program [QKHPTRC(2018)5636].

ACKNOWLEDGMENTS

We extend the sincerest appreciation to NHC Key Laboratory of Pulmonary Immunological Diseases, and Guizhou Provincial People's Hospital, for their technical assistance.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.01784/full#supplementary-material

REFERENCES

1. World Health Organization. *COVID-19*. (2020). Available online at: https://www.gisaid.org/epiflu-applications/global-cases-covid-19/ (accessed March 15, 2020).
2. Zhang JJ, Dong X, Cao YY, Yuan YD, Yang YB, Yan YQ, et al. Clinical characteristics of 140 patients infected with SARS-CoV-2 in Wuhan, China. *Allergy*. (2020) 2:19. doi: 10.1111/all.14238
3. Harcourt J, Taemin A, Lu X, Kamili S, Sakthivel SK, Murray J, et al. Severe acute respiratory syndrome coronavirus 2 from patient with 2019 novel coronavirus disease, United States. *Emerg Infect Dis*. (2020) 11:26. doi: 10.3201/eid2606.200516
4. Liu C, Jiang ZC, Shao CX, Zhang HG, Yue HM, Chen ZH, et al. A preliminary study of the relationship between novel coronavirus pneumonia and liver function damage: a multicenter study. *Zhonghua Gan Zang Bing Za Zhi*. (2020) 28:148–152. doi: 10.3760/cma.j.issn.1007-3418.2020.02.003
5. Wei ZY, Qian HY. Myocardial injury in patients with COVID-19 pneumonia. *Zhonghua Xin Xue Guan Bing Za Zhi*. (2020) 48:E006. doi: 10.1016/j.lfs.2020.117723
6. Yang X, Yu Y, Xu J, Shu H, Xia J, Liu H, et al. Clinical course and outcomes of critically ill patients with SARS-CoV-2 pneumonia in Wuhan, China: a single-centered, retrospective, observational study. *Lancet Respir Med*. (2020) 8:475–81. doi: 10.1016/S2213-2600(20)30079-5
7. Liu R, Han H, Liu F, Lv Z, Wu K, Liu Y, et al. Positive rate of RT-PCR detection of SARS-CoV-2 infection in 4,880 cases from one hospital in Wuhan, China, from Jan to Feb 2020. *Clin Chim Acta*. (2020) 505:172–175. doi: 10.1016/j.cca.2020.03.009
8. Li LQ, Huang T, Wang YQ, Wang ZP, Liang Y, Huang TB, et al. 2019 novel coronavirus patients' clinical characteristics, discharge rate, and fatality rate of meta-analysis. *J Med Virol*. (2020) 3:12. doi: 10.1002/jmv.25757
9. Dong Y, Mo X, Hu Y, Qi X, Jiang F, Jiang Z, et al. Epidemiology of COVID-19 among children in China. *Pediatrics*. (2020) 145:e20200702. doi: 10.1542/peds.2020-0702
10. Zhu N, Zhang D, Wang W, Li X, Yang B, Song J, et al. A novel coronavirus from patients with pneumonia in China, 2019. *N Engl J Med*. (2020) 20:727–33. doi: 10.1056/NEJMoa2001017
11. Lu S. Timely development of vaccines against SARS-CoV-2. *Emerg Microbes Infect*. (2020) 9:542–4. doi: 10.1080/22221751.2020.1737580
12. Nabel, GJ. HIV vaccine strategies. *Vaccine*. (2002) 15:1947. doi: 10.1016/S0264-410X(02)00074-9
13. Tomar N, De RK. Immunoinformatics: a brief review. *Methods Mol Biol*. (2014) 1184:23–55. doi: 10.1007/978-1-4939-1115-8_3
14. Oli AN, Obialor WO, Ifeanyichukwu MO, Odimegwu DC, Okoyeh JN, Emechebe GO, et al. Immunoinformatics and vaccine development: an overview. *Immunotargets Ther*. (2020) 26:13–30. doi: 10.2147/ITT.S241064
15. Baruah V, Bose S. Immunoinformatics-aided identification of T cell and B cell epitopes in the surface glycoprotein of 2019-now. *J Med Virol*. (2020) 2:5. doi: 10.1002/jmv.25698
16. Abraham PK, Srihansa T, Krupanidhi S, Vijaya SA, Venkateswarulu TC. Design of multi-epitope vaccine candidate against SARS-CoV-2: an *in-silico* study. *J Biomol Struct Dyn*. (2020) 1:1–9. doi: 10.1080/07391102.2020.1770127
17. Du L, He Y, Zhou Y, Liu S, Zheng BJ, Jiang, S. The spike protein of sars-cov a target for vaccine and therapeutic development. *Nat Rev Microbiol*. (2009) 3:226–36. doi: 10.1038/nrmicro2090
18. Walls AC, Park YJ, Tortorici MA, Wall A, McGuire AT, Veesler D. Structure, function, and antigenicity of the SARS-CoV-2 spike glycoprotein. *Cell*. (2020) 3:6. doi: 10.1101/2020.02.19.956581
19. Czub M, Weingart H, Czub S, He R, Cao J. Evaluation of modified vaccinia virus Ankara based recombinant SARS vaccine in ferrets. *Vaccine*. (2005) 23:2273–9. doi: 10.1016/j.vaccine.2005.01.033
20. Liu WL, Michael LY, Chen C. Bioinformatics analysis of sars-cov m protein provides information for vaccine development. *Progr Nat Sci*. (2003) 11:1–7. doi: 10.1080/10020070312331344530
21. Sedeyn K, Schepens B, Saelens X. Respiratory syncytial virus non-structural proteins 1 and 2: exceptional disrupters of innate immune responses. *PLoS Pathog*. (2019) 10:e1007984. doi: 10.1371/journal.ppat.1007984
22. Pandey RK, Ojha R, Mishra A, Kumar Prajapati V. Designing B-cell and T-cell multi-epitope based subunit vaccine using immunoinformatics approach to control Zika virus infection. *J Cell Biochem*. (2018) 119:7631–42. doi: 10.1002/jcb.27110
23. Pandey RK, Ali M, Ojha R, Bhatt TK, Prajapati VK. Development of multi-epitope driven subunit vaccine in secretory and membrane protein of *Plasmodium falciparum* to convey protection against malaria infection. *Vaccine*. (2018) 36:4555–65. doi: 10.1016/j.vaccine.2018.05.082
24. Pandey RK, Bhatt TK, Prajapati VK. Novel immunoinformatics approaches to design a multi-epitope subunit vaccine for malaria by investigating anopheles salivary protein. *Sci Rep*. (2018) 3:4555–65. doi: 10.1038/s41598-018-19456-1
25. Wu F, Zhao S, Yu B, Chen YM, Wang W, Song ZG, et al. A new coronavirus associated with human respiratory disease in China. *Nature*. (2020) 579:265–9. doi: 10.1038/s41586-020-2008-3
26. Zhou P, Yang XL, Wang XG, Hu B, Zhang L, Zhang W, et al. A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature*. (2020) 7798:270–3. doi: 10.1038/s41586-020-2012-7
27. Doytchinova IA, Flower DR. Identifying candidate subunit vaccines using an alignment-independent method based on principal amino acid properties. *Vaccine*. (2007) 25:866. doi: 10.1016/j.vaccine.2006.09.032
28. Doytchinova IA, Flower, DR. Vaxijen: a server for prediction of protective antigens, tumor antigens, and subunit vaccines. *BMC Bioinf*. (2007) 8:4–10. doi: 10.1186/1471-2105-8-4
29. Kelley LA, Mezulis S, Yates CM, Wass MN, Sternberg ZAMJE. The phyre2 web portal for protein modeling, prediction, and analysis. *Nat Protocol*. (2015) 10:845–58. doi: 10.1038/nprot.2015.053
30. Ko J, Park H, Heo L, Seok C. GalaxyWEB server for protein structure prediction and refinement. *Nucleic Acids Res*. (2012) 40:294–7. doi: 10.1093/nar/gks493
31. Waterhouse A, Bertoni M, Bienert S, Studer G, Tauriello G, Gumienny R, et al. SWISS-MODEL: homology modeling of protein structures and complexes. *Nucleic Acids Res*. (2018) 46:W296–303. doi: 10.1093/nar/gky427
32. Larsen MV, Lundegaard C, Lamberth K, Buus S, Lund O, Nielsen M. Large-scale validation of methods for cytotoxic t-lymphocyte epitope prediction. *BMC Bioinf*. (2007) 8:424. doi: 10.1186/1471-2105-8-424
33. Larsen M, Lundegaard C, Lamberth K, Buus S, Brunak S, Lund O. An integrative approach to CTL epitope prediction: a combined algorithm integrating MHC class I binding, TAP transport efficiency, and proteasomal cleavage predictions. *Eur J Immunol*. (2005) 35:2295–303. doi: 10.1002/eji.200425811
34. Lund O, Nielsen M, Kesmir C, Petersen AG, Lundegaard C, Worning P, et al. Definition of supertypes for HLA molecules using clustering of specificity matrices. *Immunogenetics*. (2004) 55:797–810. doi: 10.1007/s00251-004-0647-4
35. Rana A, Akhter Y. A multi-subunit based, thermodynamically stable model vaccine using combined immunoinformatics and protein structure-based approach. *Immunobiology*. (2015) 221:544–57. doi: 10.1016/j.imbio.2015.12.004
36. Nielsen M, Lundegaard C, Lund O, Can K. The role of the proteasome in generating cytotoxic t-cell epitopes: insights obtained from improved predictions of proteasomal cleavage. *Immunogenetics*. (2005) 57:33–41. doi: 10.1007/s00251-005-0781-7
37. Wang P, Sidney J, Kim Y, Sette A, Lund O, Nielsen M, et al. Peptide binding predictions for HLA DR, DP, and DQ molecules. *BMC Bioinf*. (2010) 11:568–670. doi: 10.1186/1471-2105-11-568
38. Nielsen M, Lund O. Nn-align. An artificial neural network-based alignment algorithm for MHC class ii peptide binding prediction. *BMC Bioinf*. (2009) 1:296–300. doi: 10.1186/1471-2105-10-296
39. Nielsen M, Lundegaard C, Lund, O. Prediction of MHC class ii binding affinity using SMM-align, a novel stabilization matrix alignment method. *BMC Bioinf*. (2007) 8:238. doi: 10.1186/1471-2105-8-238
40. Sidney J, Assarsson E, Moore C, Ngo S, Pinilla C, Sette A, et al. Quantitative peptide binding motifs for 19 human and mouse MHC class I molecules

derived using positional scanning combinatorial peptide libraries. *Immunome Res*. (2008) 4:2. doi: 10.1186/1745-7580-4-2
41. Sturniolo T, Bono E, Ding J, Raddrizzani L, Tuereci O, Sahin U, et al. Generation of tissue-specific and promiscuous HLA ligand databases using DNA microarrays and virtual HLA class II matrices. *Nat Biotechnol*. (1999) 17:555–61. doi: 10.1038/9858
42. Andreatta M, Karosiene E, Rasmussen M, Stryhn A, Buus S, Nielsen M. Accurate pan-specific prediction of peptide-MHC class II binding affinity with improved binding core identification. *Immunogenetics*. (2015) 67:641–50. doi: 10.1007/s00251-015-0873-y
43. Paul S, Lindestam Arlehamn CS, Scriba TJ, Dillon MB, Oseroff C, Hinz D, et al. Development and validation of a broad scheme for prediction of HLA class II-restricted T cell epitopes. *J Immunol Methods*. (2015) 422:28–34. doi: 10.1016/j.jim.2015.03.022
44. Russell CD, Unger SA, Walton M, Schwarze J. The human immune response to respiratory syncytial virus infection. *Clin Microbiol Rev*. (2017) 30:481–502. doi: 10.1128/CMR.00090-16
45. Nina C, Rupal O, Nazia, K, Kumar PV. Scrutinizing, *Mycobacterium tuberculosis* membrane and secretory proteins to formulate multiepitope subunit vaccine against pulmonary tuberculosis by utilizing immunoinformatic approaches. *Int J Biol Macromol*. (2018) 118:180–8. doi: 10.1016/j.ijbiomac.2018.06.080
46. Dhanda, SK, Vir P, Raghava GP. Designing of interferon-gamma inducing MHC class-II binders. *Biol Direct*. (2013) 8:30. doi: 10.1186/1745-6150-8-30
47. Saha S, Raghava GPS. Prediction of continuous B-cell epitopes in an antigen using recurrent neural network. *Proteins*. (2006) 65:40–8. doi: 10.1002/prot.21078
48. Jespersen MC, Peters B, Nielsen M, Marcatili P. BepiPred-2.0: improving sequence-based B-cell epitope prediction using conformational epitopes. *Nucleic Acids Res*. (2017) 45:W24–9. doi: 10.1093/nar/gkx346
49. Larsen JE, Lund O, Nielsen M. Improved method for predicting linear B-cell epitopes. *Immunome Res*. (2006) 2:2. doi: 10.1186/1745-7580-2-2
50. Chou PY, Fasman GD. Prediction of the secondary structure of proteins from their amino acid sequence. *Adv Enzymol Relat Areas Mol Biol*. (1978) 47:45–148. doi: 10.1002/9780470122921.ch2
51. Emini EA, Hughes JV, Perlow DS, Boger J. Induction of hepatitis A virus-neutralizing antibody by a virus-specific synthetic peptide. *J Virol*. (1985) 55:836–9. doi: 10.1128/JVI.55.3.836-839.1985
52. Karplus PA, Schulz GE. Prediction of chain flexibility in proteins. *Naturwissenschaften*. (1985) 72:212–3. doi: 10.1007/BF01195768
53. Kolaskar AS, Tongaonkar PC. A semi-empirical method for prediction of antigenic determinants on protein antigens. *FEBS Lett*. (1990) 276:172–4. doi: 10.1016/0014-5793(90)80535-Q
54. Parker JM, Guo D, Hodges RS. New hydrophilicity scale derived from residues with antigenicity and X-ray-derived accessible sites. *Biochemistry*. (1986) 25:5425–32. doi: 10.1021/bi00367a013
55. Tahir U, Qamar M, Saleem S, Ashfaq UA, Bari A, Anwar F, et al. Epitope-based peptide vaccine design and target site depiction against the Middle East Respiratory Syndrome Coronavirus: an immune-informatics study. *J Transl Med*. (2019) 17:362–232. doi: 10.1186/s12967-019-2116-8
56. Ferdous S, Kelm S, Baker TS, Shi J, Martin ACR. B-cell epitopes: discontinuity and conformational analysis. *Mol Immunol*. (2019) 114:643–50. doi: 10.1016/j.molimm.2019.09.014
57. Ponomarenko J, Bui HH, Li W, Fusseder N, Bourne PE, Sette A, et al. ElliPro: a new structure-based tool for the prediction of antibody epitopes. *BMC Bioinformatics*. (2008) 9:514. doi: 10.1186/1471-2105-9-514
58. Pandey RK, Ojha R, Aathmanathan VS, Krishnan M, Prajapati, VK. Immunoinformatics approaches to design a novel multiepitope subunit vaccine against HIV infection. *Vaccine*. (2018) 36:2262–72. doi: 10.1016/j.vaccine.2018.03.042
59. Nezafat N, Ghasemi Y, Javadi G, Khoshnoud MJ, Omidinia E. A novel multi-epitope peptide vaccine against cancer: an *in silico* approach. *Theor Biol*. (2014) 349:121–34. doi: 10.1016/j.jtbi.2014.01.018
60. Barh D, Misra AN, Kumar A, Azevedo V. A novel strategy of epitope design in *Neisseria gonorrhoeae*. *Bioinformation*. (2010) 5:77–82. doi: 10.6026/97320630005077
61. Mohan T, Sharma C, Bhat AA, Rao DN. Modulation of HIV peptide antigen-specific cellular immune response by synthetic α-and β-defensin peptides. *Vaccine*. (2013) 31:1707–16. doi: 10.1016/j.vaccine.2013.01.041
62. Frankel AD, Pabo CO. Cellular uptake of the tat protein from human immunodeficiency virus. *Cell*. (1988) 6:1189–93. doi: 10.1016/0092-8674(88)90263-2
63. Saha S, Raghava GP. Nucleic Acids Res. Alfred: prediction of allergenic proteins and mapping of IgE epitopes. *Mol Immunol*. (2006) 34:202–9. doi: 10.1093/nar/gkl343
64. Nicolas R, Ole L, Massimo B, Filippo C. Computational immunology meets bioinformatics: the use of prediction tools for molecular binding in the simulation of the immune system. *PLoS ONE*. (2010) 4:e9862. doi: 10.1371/journal.pone.0009862
65. Gasteiger E, Hoogland C, Gattiker A, Duval S, Wilkins MR, Appel RD, et al. Protein identification and analysis tools on the ExPASy server; the proteomics protocols handbook. *Humana Press*. (2005) 112:531–52. doi: 10.1385/1-59259-890-0:571
66. Kallberg M, Wang H, Wang S, Peng J, Wang Z, Lu H. Template-based protein structure modeling using the Phyre 2 web server. *Nat Protoc*. (2012) 7:1511–22. doi: 10.1038/nprot.2012.085
67. González-Pech RA, Stephens TG, Chan CX. Commonly misunderstood parameters of NCBI BLAST and important considerations for users. *Bioinformatics*. (2019) 15:2697–8. doi: 10.1093/bioinformatics/bty1018
68. Markus W, Manfred J. ProSA-web: interactive web service for the recognition of errors in three-dimensional structures of proteins. *Nucleic Acids Res Sippl*. (2007) 35:W407–10. doi: 10.1093/nar/gkm290
69. Vajda S, Yueh C, Beglov D, Bohnuud T, Mottarella SE, Xia B, et al. New additions to the ClusPro server motivated by CAPRI. *Proteins*. (2017) 85:435–44. doi: 10.1002/prot.25219
70. Schneidman-Duhovny D, Inbar Y, Nussinov R, Wolfson HJ. PatchDock and SymmDock: servers for rigid and symmetric docking. *Nucleic Acids Res*. (2005) 33:W363–7. doi: 10.1093/nar/gki481
71. Grote A, Hiller K, Scheer M, Munch R, Nortemann B, Hempel DC, et al. JCat: a novel tool to adapt codon usage of a target gene to its potential expression host. *Nucleic Acids Res*. (2005) 33:W526–31. doi: 10.1093/nar/gki376
72. Zuker M. Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res*. (2003) 13:3406–15. doi: 10.1093/nar/gkg595
73. Ojha R, Nandani R, Prajapati VK. Contriving multiepitope subunit vaccine by exploiting structural and non-structural viral proteins to prevent Epstein-Barr virus-associated malignancy. *J Cell Physiol*. (2019) 234:6437–48. doi: 10.1002/jcp.27380
74. Ikram A, Zaheer T, Awan FM, Obaid A, Naz A, Hanif R, et al. Exploring NS3/4A, NS5A, and NS5B proteins to design conserved subunit multi-epitope vaccine against HCV utilizing immunoinformatics approaches. *Sci Rep*. (2018) 8:16107. doi: 10.1038/s41598-018-34254-5
75. Sah R, Rodriguez-Morales AJ, Jha R, Chu DKW, Gu H, Peiris M, et al. Complete genome sequence of a 2019 novel coronavirus (SARS-CoV-2) strain isolated in Nepal. *Microbiol Resour Announc*. (2020) 9:e00169–20. doi: 10.1128/MRA.00169-20
76. Wang C, Liu Z, Chen Z, Huang X, Xu M, He T, et al. The establishment of a reference sequence for SARS-CoV-2 and variation analysis. *J Med Virol*. (2020) 3:13. doi: 10.1002/jmv.25762
77. Asaf P, Dewi H, Matthew M, Michael SR, Lakshmi S, Richard BG. Sequence-based prediction of vaccine targets for inducing T cell responses to SARS-CoV-2 utilizing the bioinformatics predictor RECON. *bioRxiv*. (2020). doi: 10.1101/2020.04.06.027805
78. Jian H, Bienfang H, Peng Z. Mimotope-based prediction of B-cell epitopes. *Methods Mol Biol*. (2014) 1184:237–43. doi: 10.1007/978-1-4939-1115-8_13
79. Karpenko O, Huang L, Dai Y. A probabilistic meta-predictor for the MHC class II binding peptides. *Immunogenetics*. (2008) 1:25–36. doi: 10.1007/s00251-007-0266-y
80. Bhattacharya M, Sharma AR, Patra P, Ghosh P, Sharma G, Patra BC, et al. Development of epitope-based peptide vaccine against novel coronavirus

2019 (SARS-COV-2): Immunoinformatics approach. *J Med Virol*. (2020) 2:28. doi: 10.1002/jmv.25736

81. Nagendra R, Hegde S, Gauthami HM, Sampath K, Jagadeesh, B. The use of databases, data mining, and immunoinformatics in vaccinology: where are we? *Exp Opin Drug Disc*. (2017) 17:117–30. doi: 10.1080/17460441.2018.1413088
82. Sikora M, Kopeć B, Piotrowska K, Pawlik A. Role of allograft inflammatory factor-1 in the pathogenesis of diseases. *Immunol Lett*. (2020) 2:1–4. doi: 10.1016/j.imlet.2019.12.002

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Medicine

SYSTEMATIC REVIEW
published: 29 July 2020
doi: 10.3389/fmed.2020.00482

Efficacy and Safety of Anti-malarial Drugs (Chloroquine and Hydroxy-Chloroquine) in Treatment of COVID-19 Infection: A Systematic Review and Meta-Analysis

Rashmi Ranjan Das[1], Nishant Jaiswal[2], Nishanth Dev[3], Nikita Jaiswal[4], Sushree Samiksha Naik[5] and Jhuma Sankar[6]**

[1] Department of Pediatrics, AIIMS Bhubaneswar, Bhubaneswar, India, [2] Evidence Based Health Informatics Unit, Department of Telemedicine, PGIMER, Chandigarh, India, [3] Department of Medicine, VMMC, Safdarjung Hospital, New Delhi, India, [4] Department of Microbiology, MM Institute of Medical Sciences and Research, MMU (Deemed to be University), Ambala, India, [5] Department of Obstetrics and Gynecology, Capital Hospital, Bhubaneswar, India, [6] Department of Pediatrics, AIIMS New Delhi, New Delhi, India

OPEN ACCESS

Edited by:
Zisis Kozlakidis, International Agency for Research on Cancer (IARC), France

Reviewed by:
Ajay Kumar Sharma, Institute of Nuclear Medicine & Allied Sciences (DRDO), India
Riccardo Pinciroli, Massachusetts General Hospital and Harvard Medical School, United States

***Correspondence:**
Rashmi Ranjan Das
rrdas05@gmail.com
Jhuma Sankar
jhumaji@gmail.com

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: 30 April 2020
Accepted: 16 July 2020
Published: 29 July 2020

Citation:
Das RR, Jaiswal N, Dev N, Jaiswal N, Naik SS and Sankar J (2020) Efficacy and Safety of Anti-malarial Drugs (Chloroquine and Hydroxy-Chloroquine) in Treatment of COVID-19 Infection: A Systematic Review and Meta-Analysis. Front. Med. 7:482. doi: 10.3389/fmed.2020.00482

Background: Anti-malarial drugs inhibit coronaviruses *in-vitro*. Few published studies have evaluated the safety and efficacy of these drugs in the treatment of COVID-19 infection.

Materials and Methods: This is a systematic review and meta-analysis of clinical trials and observational studies. Major database searches were carried out up until June 5, 2020. Participants admitted with RT-PCR-confirmed SARS Cov-2 (COVID-19) infection were included. The "Intervention group" received anti-malarial drugs with or without other drugs (Azithromycin) administered as an adjunct to the standard treatment/care. The "Control group" received treatment except anti-malarial drugs. The primary outcome is "all-cause mortality." Secondary outcome measures were effects on clinical and laboratory parameters and adverse events.

Results: Of 3,472 citations, 17 (six clinical trials and 11 observational studies) studies provided data of 8,071 participants. Compared to the control, Hydroxy-chloroquine (HCQ) has no significant effect on mortality [(OR 0.87; 95% CI 0.46–1.64); eight observational studies; $N = 5,944$]. Data from a single, small non-randomized trial ($N = 42$) also reached a similar conclusion (OR 1.94; 95% CI 0.07–50.57; $p = 0.69$). Compared to the control, HCQ plus Azithromycin (AZM) significantly increased mortality [(OR 2.84; 95% CI 2.19–3.69); four observational studies; $N = 2,310$]. Compared to the control, risk of any adverse event was significantly increased in HCQ group [(OR 3.35; 95% CI 1.58–7.13); four clinical trials; $N = 263$]. Compared to control, risk of adverse cardiac events (abnormal ECG, arrhythmia, or QT prolongation) were not significantly increased in HCQ group (but significantly increased in the HCQ plus AZM group). The GRADE evidence generated for all the outcomes was of "very low-quality."

Conclusions: As very low quality evidence suggests an increased risk of mortality and adverse event with HCQ plus Azithromycin combination (not HCQ alone), caution should

be exercised while prescribing this combination for treatment of hospitalized adults with COVID-19 infection. Good quality, multi-centric RCTs (including both hospitalized and non-hospitalized patients) are required for any firm recommendation to be made during the ongoing pandemic.

OSF Protocol Registration Link: https://osf.io/6zxsu.

Keywords: aminoquinoline, azithromycin, SARS-CoV-2, evidence-based medicine, COVID-19, mortality, Chloroquine, Hydroxychloroquine

INTRODUCTION

COVID-19, also known as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), emerged in Wuhan, China, in late 2019. It is a highly contagious disease with a global average mortality rate of 4.6% (1). There have been ongoing efforts to develop effective treatment modalities for this dreaded pandemic. Currently, no specific therapies against SARS-CoV-2 infection exist, and a series of therapeutic agents (e.g., antiviral agents, antibiotics, immune-modulators, inhaled nitric oxide, and convalescent plasma) have been repurposed with negative to inconclusive evidence available (2). There has been an increased interest in two existing anti-malarial drugs belonging to amino-quinoline group (Chloroquine and Hydroxy-chloroquine) to treat COVID-19. This is because of the inhibitory effects of these two drugs on other coronaviruses, such as SARS-CoV-1 (2, 3). The plausible mechanism of actions includes inhibition of angiotensin converting enzyme 2 (ACE-2) present on the cell surface for virus entry (by reduction of glycosylation in the enzyme) (4, 5), inhibition of release of viral particles into intra-cellular space (6, 7), and an anti-inflammatory effect (inhibition of interleukin-6, the tumor necrosis factor, the aberrant interferon, and other pro-inflammatory cytokines that cause lung injury leading to acute respiratory distress syndrome) (6, 8). Chloroquine and Hydroxy-chloroquine (HCQ) are both cost-effective and considered safe as per their approved indications. Compared to Chloroquine (CQ), Hydroxy-chloroquine (HCQ) is more soluble and less toxic and is considered safer (9, 10). It has to be kept in mind that these drugs are not entirely safe because of the risk of some serious side-effects (e.g., neuro-psychiatric, retinal, cardiac, and hypoglycemia), and there have been reports of toxicities in people who are self-medicating (11, 12).

There have been published studies evaluating the safety and/or efficacy of these agents (alone or in combination) compared to a control arm or parallel intervention, to treat patients with COVID-19 (13–32). However, the results have been contradictory. Earlier published rapid systematic reviews have concluded the role of anti-malarial drugs in patients with COVID-19 is still uncertain, and its routine use should not be recommended until more evidence is available from ongoing studies (33, 34). However, these systematic reviews neither included larger observational studies and randomized clinical trials (RCTs) published recently nor provided quality (GRADE) of evidence in a more systematic manner. In addition, findings from an ORCHID (Outcomes Related to COVID-19 treated with hydrox-ychloroquine among In-patients with symptomatic Disease) study have shown that HCQ neither harms nor benefits patients with COVID-19 infection (35). The present systematic review is an endeavor in this direction to synthesize the available evidences to inform clinical practice and guide the international agencies to formulate recommendations.

MATERIALS AND METHODS

This systematic review protocol is registered at the Open Science Forum (OSF) registration link: https://osf.io/6zxsu.

Types of Studies

Both clinical trials (randomized, quasi- randomized, and non-randomized) and observational studies comparing anti-malarial drugs (Chloroquine and Hydroxy-chloroquine) alone or in combination with other drugs vs. a control (standard of care) or other treatment were included. As a majority of the studies were published on pre-print servers (for rapid dissemination of knowledge) prior to publication in peer-reviewed journals, we planned to include these studies in the present meta-analysis after taking permission from the study authors.

Types of Participants

Children of 12–18 years of age and adults with RT-PCR-confirmed SARS Cov-2 (COVID-19) cases treated in the hospital were included. Exclusion criteria were an allergy to anti-malarial drugs [Chloroquine (CQ) and Hydroxy-chloroquine (HCQ)], retinopathy, hearing loss, and severe neuro-psychiatric diseases.

Types of Interventions

(a) Interventions included anti-malarial drugs (CQ and HCQ) provided in various formulations and dose schedules. Based on a previous study, the following dose schedules were considered: HCQ—a loading dose of 400 mg twice daily (BID) followed by a maintenance dose of 200 mg BID for 4 days; and CQ-−500 mg BID for 5 days (9). The intervention was administered as an adjunct to other treatment modalities [including Azithormycin (AZM)] to patients infected with SARS Cov-2 (COVID-19). Those in the control group received supportive treatments without CQ/HCQ. We also included trials comparing different doses (high dose vs. low-dose of anti-malarial drugs) to provide more information and urgent dissemination of knowledge during the current pandemic.

(b) Supportive and additional treatment included various methods. In hospitalized cases, it varied from bed rest,

nebulization, and oxygen inhalation to invasive respiratory support (mechanical ventilation) and maintenance of vital parameters. In addition, additional treatment during the current pandemic included antibiotics, non-specific anti-viral drugs [Remdesivir, Lopinavir/Ritonavir, IFN-α/β, Umifenovir [Arbidol], Entecavir, Ribavirin, and/or Oseltamivir], Immuno-modulators (Immunoglobulin, Tocilizumab, and Sarilumab), steroids, and NSAIDs (including Aspirin). There is evidence that non-specific antiviral drugs may not benefit patients with Covid-19, though Remdesivir and immune modulators may have some role in severe or critical cases (36, 37).

Types of Outcome Measures

Primary

1. All-cause mortality: patients with Covid-19 dying from any cause.

Secondary

1. Time to clinical recovery: time taken for normalization of temperature, respiratory distress, and relief of cough or no cough for 72 h
2. Proportion of patients with clinical recovery: proportions of patients with normalization of temperature, respiratory distress, and relief of cough or no cough for 72 h
3. Proportion of patients requiring escalation of respiratory support (including mechanical ventilation) or requiring ICU transfer: escalation of respiratory support defined as progressive change in the requirement of respiratory support to maintain normal oxygen saturation (SpO_2) and vital parameters
4. Proportion of patients developing severe disease: proportions of patients developing severe disease as defined as per the National Institute of Health (NIH) COVID-19 Treatment Guidelines (37)
5. Duration of hospitalization: the time from admission (days) to either discharge or death
6. Duration of ICU stay: the time from admission (days) to ICU to death or transfer back to non-critical areas
7. Time to negative PCR results for COVID-19: the time taken for two consecutive negative reports of a positive patient
8. Proportion of patients with negative PCR results for COVID-19 after day 3, 5, 7, 10, 14, 21, and 28: proportions of patients with two consecutive negative reports after a positive report
9. Proportion of patients with improved radiological features after day 3, 5, 7, 10, 14, 21, and 28: proportions of patients with improvement noted in either chest X ray or CT scan of chest compared to that done at baseline
10. Effect on hematological parameters (including inflammatory markers): these include the blood parameters (complete blood count, differential counts, and platelet count), acute phase reactants (ESR, CRP, and pro-calcitonin), and inflammatory markers (IL-6, TNF-α, etc.)
11. Adverse events: developing secondary to the use of anti-malarial drugs alone or in combination with other drugs.

Search Methodology

The following major databases were searched systematically from 1970 till June 5, 2020: Cochrane Central Register of Controlled Trials (CENTRAL), PubMed/MEDLINE, Google Scholar, and EMBASE (**Appendix 1**). We also searched the Pre-print servers (medRxiv, bioRxiv, OSF pre-prints, Pre-prints.org) till June 5, 2020. The PubMed/Medline search strategy used the various MeSH and free text terms for "novel corona virus," "COVID 19," "Hydroxychloroquine," and "Chloroquine" combined using the Boolean operators. No language restrictions were applied. Three reviewers (RRD, NJ, and ND) reviewed the search results to identify relevant studies.

Data Extraction

Data extraction was done using a data extraction form that was designed and pilot tested a priori. Three authors (NJ, ND, and SSN) independently extracted the following information from each study: author; year; location (country); study design (clinical trial or observational study); setting (hospital or community); method of recruitment; inclusion criteria; unit of analysis; allocation ratio In case of RCT); risk of bias; participants (age, sex, sample size, and disease severity); intervention (dosage, duration, frequency, and co-intervention if any); outcomes (outcome definition, valid unit of measurement, time points of collection and reporting); loss to follow-up; and miscellaneous (key conclusions, references to other relevant trials, and additional data required).

Assessment of Risk of Bias in the Included Studies

Two review authors independently (NJ and SSN) assessed the methodological quality of the selected trials by using methodological quality assessment forms and the criteria outlined in the Cochrane Handbook for Systematic Reviews of Interventions (38). Quality assessment was undertaken using the Newcastle Ottawa Scale (NOS) for observational studies. This scale assesses the quality under three major headings, namely, selection of the studies (representativeness and the exposure assessment/control selection), comparability (adjustment for main/additional confounders), and outcome/exposure (adequacy of outcome measured, exposure measured vs. self-report) (39). Quality assessment was undertaken using the ROBINS-I tool for non-randomized trials (40). Any disagreements between the two review authors were resolved through discussion with a third author (JS).

Dealing With Missing Data

We described missing data, including dropouts in included studies. Differential dropout rates can lead to biased estimates of the effect size, and bias may arise if the reasons for dropping out differ across groups. We reported reasons participants dropped out of studies as mentioned by the authors. If data were missing, or if reasons for dropping out were not reported, we contacted the authors for further information.

Data Synthesis

Data were analyzed using Review Manager (RevMan) V.5.1 (The Nordic Cochrane Center, The Cochrane Collaboration,

Copenhagen, Denmark) (41). The data from various studies were pooled and expressed as mean difference (MD) with 95% confidence interval (CI) in case of continuous data, and odds ratio (OR) with 95% CI in case of categorical data. Where data were expressed as a median (IQR), we calculated the mean and SD by the statistical formula described previously (42). The primary pooled analysis of all the reports was conducted using the Generic Inverse Variance method using random effects weighting (43), where the log RRs for cohort studies or log ORs for case–control studies were weighted by the inverse of the variance to obtain a pooled RR estimate. Since nested case-cohort and nested case–control studies are temporally prospective, we analyzed data from these studies with the prospective studies. A $p < 0.05$ was considered statistically significant. Inter-study heterogeneity was assessed by Cochrane's Q (χ^2 $p < 0.10$) and quantified by I^2. An $I^2 \geq 50\%$ indicated "substantial" heterogeneity and ≥75% indicated "considerable" heterogeneity (44). The cause of substantial and considerable heterogeneity was explored, and sensitivity and/or sub-group analyses were carried out.

Publication Bias

To evaluate for any possible publication bias, we constructed the funnel plot from primary outcome data (45).

Grade of Evidence

To assess the quality of evidence we used GRADE Profiler software (V.3.2) (46, 47). The software uses five parameters for rating the quality of evidence. The parameters used were limitations to design of randomized controlled trials, inconsistency of results or unexplained heterogeneity, indirectness of evidence, imprecision of results, and publication bias. The rating was determined as no, serious, or very serious limitations.

RESULTS

Description of Studies

Of 3,472 total citations retrieved, the full texts of 49 papers were assessed for eligibility, and 29 were excluded for various reasons (**Figure 1**). Of the remaining 20 eligible studies, 14 were published in peer-reviewed journals (13–29) and six in pre-print servers (not peer-reviewed) (17, 28–32). We contacted the authors of these six studies to give us their permission to use their data in the meta-analysis, but only three authors gave their permission (17, 28, 29). We therefore included the data of three studies in the meta-analysis and described the characteristics of the remaining three studies using a separate table (**Supplementary Table 1**). Finally, we were able to conduct a meta-analysis of a total of 17 studies (six clinical trials and 11 observational studies) including 8,071 patients (Adults = 8,041; Adolescents = 30) (**Table 1**). Twenty-nine studies were excluded for the following reasons: 19 were case series (without having a control/comparator that is inclusion criteria of present review), nine studies mentioned about intervention but did not provide outcome data for them separately, and one study reported use of anti-malarial drugs with or without AZM in rheumatoid arthritis (RA) patients for non-RA indications (including viral and other infections).

Of 17 published peer-reviewed studies included, six clinical trials provide data of 381 patients, and the 11 observational studies provided data of 8,071 patients. A total of 4,009 patients received HCQ or CQ (clinical trials = 226, observational studies = 3,783), and 1,255 received a combination of HCQ plus Azithromycin (clinical trials = 06, observational studies = 1,249). The studies were conducted in following countries: USA (five studies, 3,985 patients), Spain (two studies, 2,185 patients), China (four studies, 752 patients), France (two studies, 217 patients), Brazil (one study, 81 patients), and the UAE (one study, 34 patients). One trial compared high vs. low-dose of Chloroquine (18). One clinical trial (13) and six observational studies (19–22, 25, 27); each had three arms of comparison (HCQ, HCQ+AZM, and Control). Two studies included data on adolescents (<18 years) (13, 21). Two studies used Azithromycin but did not provide separate outcome data for both the groups (19, 20). Of the six clinical trials, three were described as double blinded, two were open label, and one was a non-randomized trial.

As shown in **Table 1**, the age of included participants, severity of illness, dose schedule, and timing of administration of intervention (HCQ/CQ) varied widely among the studies. Majority of the participants in the clinical trials were ≤50 yr of age, whereas, majority of the participants in the observational studies (except one) were ≥60 yr of age. Around 72% of participants in the clinical trials were having mild and moderate illness, whereas <40% of the participants in the observational studies were having mild and moderate illness. One study included only cancer patients (27). The dose of CQ was nearly uniform (except one RCT comparing high and low-dose) with duration varying from 5 to 10 days. The dose of HCQ varied widely with the lowest dose being 200 mg/d−1,200 mg on day 1 followed by variable doses for variable period (sometime till discharge/death). Two studies did not provide any information on dose schedule of HCQ (26, 27). The median time from onset of symptom to admission or treatment initiation was ≤8 days in all but two studies (one RCT has 17 days, and one observational study has 10 days). Two studies did not provide any information on the timing of initiation of HCQ (26, 27). Except one study (17), no other study was able to start the intervention (HCQ/CQ) in the early phase of illness (within 48 h of symptom onset), which is regarded as the golden window for antiviral treatment (e.g., in influenza) (48).

Risk of Bias in Included Studies

The details have been provided in **Appendix 2**. Except two trials (17, 18), others had low to high-risk of bias in different domains. One non-randomized trial had a serious risk of bias overall (13). Of the 11 observational studies, five were at a high risk of bias for selection of cases (22, 23, 25, 28, 29). Except for one study (27), the remaining 10 studies were at a high risk of bias for selection of controls and a low risk of bias for the exposure parameters.

Effect of Interventions

Primary Outcomes (All-Cause Mortality)

HCQ vs. control

(i) overall results: Three trials reported no mortality in any of the groups. One Non-RCT ($N = 42$) found no significant difference in the mortality rate between HCQ and control

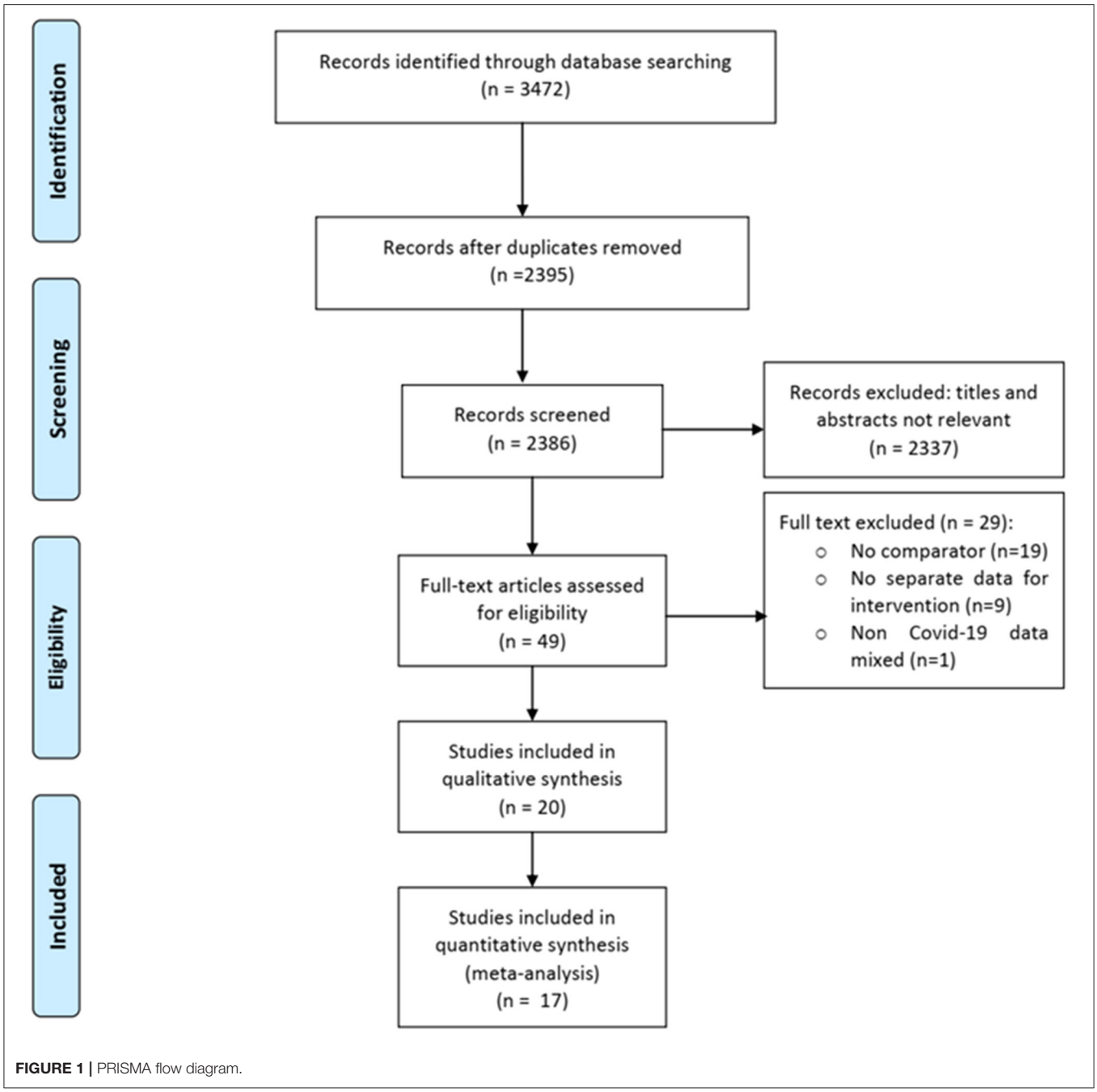


FIGURE 1 | PRISMA flow diagram.

group (OR 1.94; 95% CI 0.07–50.57; $p = 0.69$) (13) (**Supplementary Figure 1**). Eight observational studies ($N = 5,944$) reported mortality rate, and found no significant difference between the HCQ and control group (OR 0.87; 95% CI 0.46–1.64; $p = 0.66$; $I^2 = 92\%$) (**Figure 2**) (19–21, 23, 25–27, 29).

(ii) Subgroup analysis (data from observational studies): Mortality rate was found to be significantly increased in the HCQ group in the studies from USA (OR 1.71; 95% CI 1.38–2.13; $p < 0.001$; $I^2 = 0\%$; $N = 3,036$) (20, 21, 25, 27), whereas a significantly decreased mortality rate was found in the studies conducted outside USA ($N = 2,908$) population (OR 0.38; 95% CI 0.23–0.63; $p < 0.001$; $I^2 = 56\%$) (19, 23, 26, 29). The heterogeneity was not significant once we separated studies conducted in USA vs. outside USA. Two studies (20, 23) compared mortality rate in participants aged ≤60 vs. >60 yr and found significantly increased risk in those >60 yr age [data provided as hazard ratio [not raw data]]. One study used HCQ after 48 h of admission and two studies had no information on timing; when these two studies were omitted, no difference in mortality was found (OR 1.24; 95% CI 0.7–2.18; $p = 0.46$; $I^2 = 82\%$) (23, 26, 27). When studies

TABLE 1 | Characteristics of included studies.

Study author, Country (Reference)	Sample size (*N*), additional inclusion criteria*	Age and sex of participants	Illness severity of participants	Intervention group (dose schedule)	Time from onset of symptom to treatment (d)	Supportive and additional treatment used	Additional comments
CLINICAL TRIALS (RANDOMIZED AND NON-RANDOMIZED)							
Gautret et al. France (single center) (13)	*N*: 36 (HCQ = 14; HCQ+AZM = 6; Control =16). Additional inclusion criteria: None.	Age (yr): >12 yr (HCQ = 51.2 ± 18.7; Control = 37.3 ± 24). Male: 41.7%.	All severity included. Asymptomatic: 16.7% URTI: 61.1% LRTI: 22.2%.	HCQ: 600 mg/d (200 mg TID) for 10 days. HCQ+AZM: AZM 500 mg on day 1 followed by 250 mg OD for 4 days in addition to HCQ.	Mean (SD): 4.1 (2.6) in HCQ group, and 3.9 (2.8) in Control group.	Symptomatic and antibiotics.	HCQ group recruited in one center and control group in another. Control group included those refused intervention or were not eligible for it. Attrition rate 23% in HCQ group. Funded study. There were protocol deviations.
Chen et al. China (single center) (14)	*N*: 30 (HCQ = 15; Control = 15). Additional inclusion criteria: None.	Age (yr): >18 yr (HCQ = 50.5 ± 3.8; Control = 46.7 ± 3.6). Male: 70%.	Severe illness or other measures of severity not defined.	HCQ: 400 mg/d (OD) for 5 days.	Not mentioned.	Respiratory support and others (anti-virals, IFN-α, nebulisation, and antibiotics). Arbidol (Umifenovir): HCQ group (80%), Control group (67%). Lopinavir/Ritonavir: HCQ group (0%), Control group (13%).	Underlying co-morbidities: hypertension (27%), diabetes (7%), and chronic obstructive lung disease (3.5%). Started enrolment 1 day prior to trial registration. Funded study.
Tang et al. China (multi-center) (15)	*N*: 150 (HCQ = 75; Control = 75). Additional inclusion criteria: A Chest CT scan needed before randomization.	Age (yr): >18 yr (HCQ = 48.0 ± 14.1; Control = 44.1 ± 15.0). Male: 54.7%.	Mild: 14.7% Moderate: 84% Severe: 1.3%.	HCQ: 1,200 mg/d for 3 days followed by 800 mg/d for the remaining days (total treatment duration: 2 weeks for mild/moderate and 3 weeks for severe cases).	Mean: 16.6 (HCQ started within 24 h of randomization).	Respiratory support. ntibiotics (39%), anti-virals (Lopinavir/Ritonavir, Arbidol, Ribavirin, and/or Oseltamivir), and Steroid (7%).	Trial stopped early (intended to enroll 360 patients-−180 in each arm). Underlying co-morbidities (30%): diabetes (14%), hypertension (6%), and others (20.7%). Funded study. Shanghai Pharma donated HCQ.
Huang et al. China (single center) (16)	*N*: 22 (CQ = 10; Control = 12). Additional inclusion criteria: None.	Age (yr): >18 yr (CQ [median, IQR] = 41.5 [33.8–50]; Control [median, IQR] = 53 [41.8–63.5]). Male: 59.1%.	Moderate: 64% Severe: 36%.	CQ: 1,000 mg/d (500 mg BID) for 10 days. Lopinavir (400 mg)/Ritonavir (100 mg): BID for 10 days in the control group.	Median: 2.5 in CQ group, and 6.5 in Control group.	Respiratory support, antibiotics, anti-virals, and steroid.	Underlying co-morbidities: hypertension (18.2%), diabetes (9.1%), smoking (9.1%), and cerebro-vascular disease (4.5%). No protocol deviation. Funding status not mentioned.
Chen et al. China (single center) (17)	*N*: 62 (CQ = 31; Control = 31). Additional inclusion criteria: Chest CT with pneumonia; SaO_2/SPO_2 ratio > 93% or PaO_2/FIO_2 ratio > 300 mmHg (mild illness).	Age (yr): >18 yr (HCQ = 44.1 ± 16.1; Control = 45.2 ± 14.7). Male: 46.8%.	Mild: 100%	HCQ: 400 mg/d (200 mg BID) for 5 days.	Both groups had fever and cough 1 day before starting of intervention (intervention started within 48 h)	Oxygen therapy, antiviral agents, antibacterial agents, and Immunoglobulin, with or without Corticosteroids.	No information on underlying co-morbidities. Significant deviation from registered protocol. Funded study. Shanghai Pharma provided the HCQ tablets.

(Continued)

TABLE 1 | Continued

Study author, Country (Reference)	Sample size (*N*), additional inclusion criteria*	Age and sex of participants	Illness severity of participants	Intervention group (dose schedule)	Time from onset of symptom to treatment (d)	Supportive and additional treatment used	Additional comments
Borba et al. Brazil (single center) (18)	*N*: 81 (CQ high-dose = 41; CQ low-dose = 40). Additional inclusion criteria: RR >24/min and/or HR >125 bpm and/or SpO_2 <90% in ambient air and/or shock.	Age (yr): >18 yr (CQ high-dose = 54.7 ± 13.7; CQ low-dose = 47.4 ± 13.3). Male: 75.3%.	Severe: 89% (33% were critical)	High-dose CQ: 600 mg BID for 10 days (total dose 12 g). Low-dose CQ: 450 mg BID on day 1 followed by OD for 4 days (total dose 2.7 g).		Ceftriaxone (7 days) plus azithromycin (5 days) in all cases, and Oseltamivir (5 days) in 87% cases.	Co-morbidities: hypertension (45.5%), alcohol disorder (27.5%), and diabetes (25.5%). Older and more heart disease (high-dose = 17.9%, low-dose = 0) in the high-dose group. Funded study.
OBSERVATIONAL STUDIES							
Mahévas et al. France (multi-center) (19)	*N*: 181 (HCQ = 84; Control = 97). Additional inclusion criteria: requiring oxygen by mask or nasal prongs (WHO progression scores of 5).	Age (yr): >18 yr (HCQ [median, IQR] = 59 [48–67]; Control [median, IQR] = 62 [55–69]. Male: 72%.	Severe: 100%.	HCQ: 600 mg/d	Median: 7 (HCQ started within 24 h of admission except in 8 cases).	Respiratory support, Azithromycin (HCQ = 18%, Control = 29%); Amoxicillin and Clavulanic acid (HCQ = 52%, Control = 28%). No patient received anti-viral drugs or anti-inflammatory drugs.	Co-morbidities: cardio-vascular disease (55%), obesity (26%), immunosuppression (12%), chronic respiratory disease (11%), diabetes (9%), and chronic kidney disease (5%). Virological cure (repeat PCR) not checked. Non-funded study.
Geleris et al. USA (single center) (20)	*N*: 1,376 (CQ = 811; Control = 565). Additional inclusion criteria: None.	Age (yr): >18 yr [Majority were ≥60 years of age (60.5%)]. Male: 56.8%.	Severe illness or other measures of severity not defined (HCQ group were more severely ill than control group).	HCQ: 600 mg BID on day 1 followed by 400 mg OD for 4 days.	Not mentioned (in 86% cases, HCQ started within 48 h of admission).	Respiratory support, antibiotics (66.1%), Azithromycin (44.5%), Tocilizumab (5.1%), Remdesivir (2%), Sarilumab (2.2%), and Corticosteroids (19.8%).	Co-morbidities: diabetes (35.7%), hypertension (31.7%), chronic lung disease (18.2%), chronic kidney disease (17.3%), cancer (12.8%), smoking (11.4%), and transplant/HIV/immunosuppression (4.2%).
Rosenberg et al. USA (multi-center) (21)	*N*: 1,438 (HCQ = 271; HCQ+AZM = 735; AZM = 211; Control = 221). Additional inclusion criteria: None.	Age (yr) (median): Children and Adults (HCQ = 65.5; HCQ+AZM = 61.4; Control = 64). Male: 59.7%.	All severity included (HCQ group: 30% critically ill; Control group: 10% critically ill). Only HCQ group had the highest levels of chronic lung disease (25.1%) and cardiovascular conditions (36.5%). Obese, diabetes, dementia Black or Hispanic patients, clinically severity score and abnormal radiological findings were significantly more in HCQ group.	HCQ: 200–600 mg in OD or BID schedule (variably used).	Median: three in the HCQ group, two in the HCQ+AZM group, and four in the Control group (HCQ started within 48 h of admission).	Respiratory support, Aspirin (19.8%), and NSAIDs (3.6%).	Included 25 children. Co-morbidities: diabetes (35%), obesity (30.4%), cardio-vascular disease (30.4%), chronic lung disease (18%), smoking (17.4%), kidney disease (13%), dementia (6.5%), and cancer (3.8%). Patients entered the ICU/mechanical ventilated, often with HCQ and AZM initiation, rendered these outcomes unsuitable for efficacy analyses. Adverse events were collected, potentially before drug initiation. Conflict of interest unclear (spouse of one author received grant from Gilead foundation). Funded study.

(Continued)

TABLE 1 | Continued

Study author, Country (Reference)	Sample size (*N*), additional inclusion criteria*	Age and sex of participants	Illness severity of participants	Intervention group (dose schedule)	Time from onset of symptom to treatment (d)	Supportive and additional treatment used	Additional comments
Saleh et al. USA (single center) (22)	*N*: 201 (CQ/HCQ = 82; CQ/HCQ+AZM = 119). Additional inclusion criteria: None.	Age (yr): >18 yr (mean ± SD = 58.5±9.1). Male: 57.2%.	Severe illness or other measures of severity not defined.	CQ/HCQ: CQ 500 mg BID on day 1 followed by OD for 4 days; HCQ 400 mg BID on day 1 followed by 200 mg BID for 4 days (total 5 days. HCQ+AZM: AZM 500 mg OD for 5 days in addition to above.	Not mentioned.	Respiratory support.	Co-morbidities: hypertension (60.2%), hyperlipidemia (41.8%), diabetes (32.3%), chronic lung diseases (14.9%), coronary artery disease (11.4%), heart failure (7.5%), atrial fibrillation (7%), and chronic kidney disease (5%). No virological outcome studied. Non-funded study.
Yu et al. China (Single- center) (23)	*N*: 550 (HCQ = 48; Control = 502). Additional inclusion criteria: CT chest suggestive and critically ill (corresponding to a WHO progression score of 5).	Age (yr) [median (IQR)]: >18 yr [HCQ = 68 (60–75); Control = 68 (59–77)]. Male: 62.5%.	Critically ill (100%).	HCQ: 400 mg/d (200 mg BID for 7–10 days).	Median (IQR): 10 (3–13) after admission.	Respiratory support. antivirals (Lopinavir/Ritonavir, Entecavir hydrate, or Ribavirin), IVIg, antibiotics, and Interferon (no Interferon in HCQ group).	Co-morbidities were: hypertension (45.8%), diabetes (17.1%), coronary heart disease (10.7%), and COPD (2.9%). Funded study.
Huang et al. China (Multi-center) (24)	*N*: 373 (CQ = 197; Control = 176). Additional inclusion criteria: None.	Age (yr) [median (IQR)]: >18 yr [CQ = 43 (33–55); Control = 47.5 (35.8–56)]. Male: 46.9%.	Mid: 3.8% Moderate: 91.4% Severe: 4.8%.	CQ: 500–1,000 mg/d (OD or BID) for 10 days.	Median (IQR): 7 (3–10.8) after admission (Guangdong province). Median (IQR): 19 (17–124.5) after admission (Hubei province).	Respiratory support. Only Control group received following treatment: antivirals (Arbidol, Lopinavir/Ritonavir), Chinese traditional medicine, and Interferon.	Co-morbidities were: hypertension (6.4%) and diabetes (2.4%). Funded study.
Magagnoli et al. USA (single center) (25)	*N*: 807 (HCQ = 198; HCQ+AZM = 214; Control = 395). Additional inclusion criteria: Availability of data on body mass index, vital parameters.	Age (yr) [median (IQR)]: >18 yr [HCQ = 71 (62–76.8); HCQ+AZM = 68 (59–74); Control = 70 (59–77)]. Male: 95.7%	All severity included (no severity subgroups mentioned).	HCQ [median (IQR) daily dose]: 400 (400–480) mg in HCQ group, and 422.2 (400–480) mg in HC+AZM group for median (IQR duration of 5 (3–6) d.	Not mentioned (HCQ and AZM started within 24 h).	Respiratory support, and Azithromycin (23% in control group only).	Co-morbidities: Diabetes mellitus (66.2%), cardio-vascular disease (42.9%), renal disease (25%), chronic pulmonary disease (19.6%), malignancy (18%), hyper-lipidemia (15.8%), cerebro-vascular diseases (15%), smoking (14.1%), liver disease (9.2%), dementia (8.4%), asthma (6%), and HIV/AIDS (2.4%). There were significant differences among the three groups in baseline demographic characteristics, selected vital signs, laboratory tests, prescription drug use, and co-morbidities.

(Continued)

TABLE 1 | Continued

Study author, Country (Reference)	Sample size (*N*), additional inclusion criteria*	Age and sex of participants	Illness severity of participants	Intervention group (dose schedule)	Time from onset of symptom to treatment (d)	Supportive and additional treatment used	Additional comments
Ayerbe et al. Spain (multi-center) (26)	Number: 2,019 (HCQ = 1,857; Control = 162). Additional inclusion criteria: None.	Age (yr): >18 yr (HCQ = 67.11 ± 15.51; Control = 73.47 ± 16.22) Male: 57.3%.	All severity included (no severity subgroups mentioned).	HCQ: dose and schedule not mentioned.	Not mentioned.	Respiratory support, anti-virals (Lopinavir/Ritonavir), Tocilizumab, Steroids, Heparin, and Oseltamivir.	No information on underlying co-morbidities. Funded study.
Kuderer et al. USA, Canada, and Spain (multi- center) (27)	Number: 756 (HCQ = 89; HCQ+AZM = 181; Control = 486). Additional inclusion criteria: underlying malignancy.	Age (yr) [median (IQR)]: >18 yr [66 (57–76)]. Male: 50%.	All severity included (no severity subgroups mentioned).	HCQ: dose and schedule not mentioned.	Not mentioned.	Not mentioned except for the specific treatment of malignancy	Co-morbidities: Malignancy (100%), and obesity (19%). Funded study. HCQ+AZM was given to patients with severe illness.
Mallat et al. UAE (single center) (28)	N: 34 (HCQ = 23; Control = 11). Additional inclusion criteria: None.	Age (yr): [median (IQR)]: >18 yr [HCQ = 33 (31 – 48); Control = 41 (30–55)]. Male: 73.5%.	Mild and moderate (100%).	HCQ: 800 mg/d (400 mg BID) on day 1 400 mg/d for 10 days.	Median: 4 (HCQ started within 24 h).	Respiratory support. Others not mentioned.	Co-morbidities: hypertension (14.7%), asthma (8.8%), diabetes (5.9%), heart disease (2.9%), renal disease (2.9%), and immunosuppressant use (2.9%). Co-morbidities and D-dimer levels were significantly higher in the non-HCQ group.
Membrillo et al. Spain (single center) (29)	Number: 166 (HCQ = 123; Control = 43). Additional inclusion criteria: bilateral pneumonia with clinical picture compatible with COVID-19.	Age (yr): >18 yr (HCQ = 61.5 ± 16.2; Control = 68.7 ± 18.8). Male: 62%.	Mild: 50% Moderate: 29% Severe: 21%.	HCQ: 1,200 mg (800 mg + 400 mg) loading dose on day 1 followed by 400 mg OD.	Median: 7 in HCQ group (started within 24 h).	Respiratory support, anti-virals (Lopinavir/Ritonavir), IFN-β), and/or anti-inflammatory drugs (steroids and/or tocilizumab).	Co-morbidities: hypertension (42.8%), dyslipidemia (34.3%), heart disease (22.3%), diabetes (17.5%), cancer (13.9%), and pulmonary disease (14.4%).

HCQ, Hydroxychloroquine; CQ, Chloroquine; AZM, Azithromycin; RT-PCR, Reverse transcription polymerase chain reaction; MV, Mechanical ventilation; COPD, Chronic obstructive pulmonary disease; URTI, Upper respiratory tract infection; LRTI, Lower respiratory tract infection; ARDS, Acute respiratory distress syndrome; IQR, Inter-quartile range; ICU, Intensive care unit; WHO, World health organization; OD, Once daily; BID, Twice daily; NSAIDs, Non-steroidal anti-inflammatory drugs; IVIg, intravenous immunoglobulin.

**Additional inclusion criteria, any additional features besides RT-PCR positive SARS-CoV-2.*

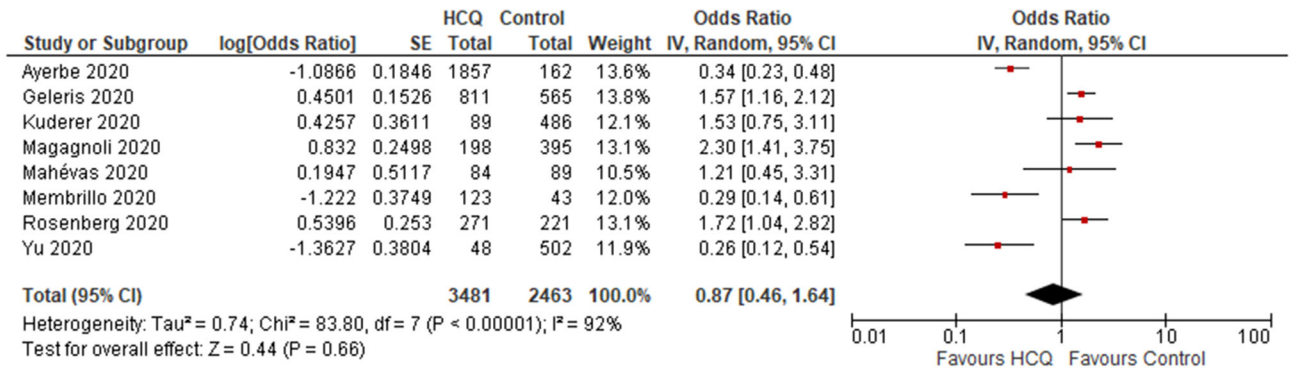


FIGURE 2 | All-cause mortality (HCQ vs. control; observational studies).

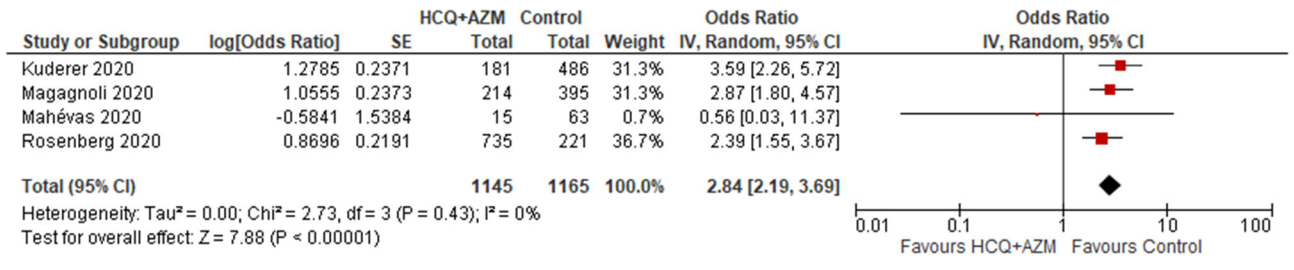


FIGURE 3 | All-cause mortality (HCQ + AZM vs. control; observational studies).

with median time from onset of symptom to admission or treatment initiation of >8 days were excluded, and no significant difference was found (OR 1.24; 95% CI 0.7–2.18; $p = 0.46$; $I^2 = 82\%$). We could not carry out subgroup analyses of mortality rate in participants with and without co-morbidity, as these data were not provided separately by the included studies. When studies that did not follow the recommended dose schedule of HCQ/CQ were excluded, still no significant difference was found (OR 0.83; 95% CI 0.36–1.88; $p = 0.65$; $I^2 = 90\%$).

HCQ plus azithromycin (AZM)

Four studies ($N = 2{,}310$) reported a significant increase in the mortality rate in the HCQ plus AZM group compared to the control group (OR 2.84; 95% CI 2.19–3.69; $p < 0.001$) (19, 21, 25, 27) (**Figure 3**). Another study used Azithromycin in treatment but did not provide separate data (19).

HCQ vs. HCQ plus AZM

Five studies ($N = 1{,}988$) reported mortality rate, and found a significant decrease in HCQ group (OR 0.7; 95% CI 0.54–0.9; $p = 0.006$; $I^2 = 0\%$) (19, 21, 22, 25, 27).

High-dose vs. low-dose CQ

One RCT ($N = 81$) found a significantly higher mortality rate in the high-dose group (OR 3.63; 95% CI 1.24–10.58; $p = 0.02$) (**Supplementary Figure 2**) (18).

Secondary Outcomes

Details have been provided in **Table 2**. A majority of the outcome measures favored the Control group (i.e., the Control was better than HCQ±AZM), and these were the occurrence of adverse events [any events (**Supplementary Figure 3**), or only cardiac events, or only vomiting], development of severe disease, and duration of hospitalization. Those favored HCQ group (i.e., HCQ±AZM was better than Control) were resolution of cough, proportion of patients with negative COVID-19 PCR after days 5, 10, and 14, proportion of patients with improved radiological features after day 5, change in IL-6 level (pg/mL), and change in total leukocyte count (/cumm). The outcomes that favored HCQ over HCQ plus AZM were mortality rate and the development of severe disease. Contrary to common belief, no difference between HCQ and HCQ plus AZM was found for any type of adverse cardiac events.

Publication Bias

The funnel plot was asymmetrical showing publication bias (**Supplementary Figure 4**). The reasons for publication bias were heterogeneity among studies, poor methodological design, and selective outcome reporting.

Grade of Evidence

The evidence generated was of "very low quality" for all the outcomes (primary and secondary). A detailed analysis of the summary of evidence is provided in **Table 3**.

TABLE 2 | Secondary outcome measures from the included studies.

Name of outcome	No of trials (Reference)	Sample size	Effect estimate	*P*-value
CLINICAL TRIALS (RANDOMIZED, AND NON-RANDOMIZED)				
Hydroxy-chloroquine (HCQ)/chloroquine (CQ) vs. control				
Time to alleviation of clinical symptoms (d)				
Fever	2 (14, 17)	92	MD 0.21; 95% CI −2.95 to 3.37	0.9
Cough	1 (17)	62	MD −1.1; 95% CI (−1.86 to −0.34)	0.005*
Clinical recovery	1 (15)	119	Could not be pooled	0.96
Time to negative RT- PCR results (d)	2 (14, 15)	180	MD 1.55; 95% CI −0.7 to 3.79	0.18
Escalation of respiratory support (including MV)	1 (13)	42	OR 4.92; 95% CI 0.24 to 101.66	0.3
Development of severe disease	1 (17)	62	OR 0.1; 95% CI 0.0 to 1.88	0.12
Proportion with clinical recovery after day 28	1 (15)	150	OR 0.75; 95% CI 0.39 to 1.46	0.34
Proportion with negative RT- PCR				
After day 3	2 (13, 15)	180	OR 1.02; 95% CI 0.16 to 6.6	0.98
After day 5	1 (13)	30	OR 9.33; 95% CI 1.51 to 57.65	0.02*
After day 7	3 (14–16)	202	OR 0.65; 95% CI 0.36 to 1.17	0.15
After day 10	1 (15)	150	OR 0.73; 95% CI 0.37 to 1.47	0.38
After day 14	3 (14–16)	202	OR 0.98; 95% CI 0.44 to 2.15	0.95
After day 21	1 (15)	150	OR 1.49; 95% CI 0.62 to 3.61	0.37
After day 28	1 (15)	150	Not pooled (event NE in HCQ group)	
Proportion with improved radiological features				
After day 3	1 (14)	30	OR 0.57; 95% CI 0.13 to 2.5	0.46
After day 5	1 (17)	62	OR 3.43; 95% CI 1.1 to 10.7	0.03*
After day 14	1 (14)	30	All patients (HCQ and control group) improved	
Adverse events				
Any	4 (14–17)	263	OR 3.35; 95% CI 1.58 to 7.13	0.002*
Serious	1 (15)	150	OR 5.88; 95% CI 0.28 to 124.5	0.26
Vomiting	2 (15, 16)	172	OR 8.67; 95% CI 1.32 to 56.99	0.02*
Abdominal complaints	2 (15, 16)	172	OR 0.77; 95% CI 0.12 to 5.11	0.79
Diarrhea	3 (14–16)	202	OR 2.45; 95% CI 0.25 to 24.18	0.44
Transamnitis	2 (14, 15)	180	OR 1.74; 95% CI 0.2 to 14.78	0.61
Kidney injury	2 (14, 15)	180	OR 1.06; 95% CI 0.1 to 11.3	0.96
Hydroxy-chloroquine (HCQ) and azithromycin (AZM) vs. control				
Proportion of patients with negative RT-PCR				
After day 3	1 (13)	22	OR 15.0; 95% CI 1.32 to 169.89	0.03*
After day 5	1 (13)	22	OR 0.45; 95% CI 0.02 to 10.67	0.62
High-dose vs. low-dose chloroquine (CQ)				
Proportion of patients with negative RT-PCR				
After day 3	1 (18)	27	No separate data (six patients negative)	NE
Adverse events	1 (18)	81	OR 2.27; 95% CI 1.14 to 4.49	0.02*

Name of outcome	No of studies (Reference)	Sample size	Effect estimate	*P*-value
OBSERVATIONAL STUDIES				
Hydroxy-chloroquine (HCQ) or Chloroquine (CQ) vs. control				
Escalation of respiratory support (including MV)	5 (19–21, 25, 27)	3,247	OR 2.04; 95% CI 0.99 to 4.18	0.05
Development of severe disease	3 (19, 21, 24)	1,038	OR 1.12; 95% CI 0.51 to 2.46	0.77
Duration of hospitalization (d)	5 (21, 23–25, 29)	1,858	MD 2.17; 95% CI 0.21 to 4.13	0.03*
Time to negative RT- PCR results (d)	2 (24, 28)	407	MD 1.14; 95% CI −11.98 to 14.26	0.86

(Continued)

TABLE 2 | Continued

Name of outcome	No of studies (Reference)	Sample size	Effect estimate	*P*-value
Proportion of patients with negative RT- PCR				
After day 10	1 (24)	373	OR 7.86; 95% CI 4.4 to 14.04	<0.001*
After day 14	2 (24, 28)	407	OR 6.37; 95% CI 3.01 to 13.48	<0.001*
Proportion with improved radiological features				
After day 10	1 (24)	71	OR 1.13; 95% CI 0.38 to 3.3	0.83
After day 14	1 (24)	71	OR 0.88; 95% CI 0.32 to 2.46	0.81
Effect on hematological parameters				
Change in IL-6 level (pg/mL)	1 (23)	550	MD −20.64; 95% CI −26.24 to −15.04	<0.001*
Change in CRP level (mg/L)	1 (28)	34	MD −4.95; 95% CI −34.17 to 24.27	0.74
Change in total leukocyte count (/cumm)	1 (28)	34	MD −1247.7; 95% CI −2356.6 to −138.7	0.03*
Change in total lymphocyte count (/cumm)	1 (28)	34	MD −190.75; 95% CI −998.12 to 616.62	0.64
Change in serum ferritin (μg/L)	1 (28)	34	MD −165.97; 95% CI −680.53 to 348.59	0.53
Adverse events				
Any	1 (24)	373	OR 0.77; 95% CI 0.49 to 1.2	0.25
Abnormal ECG	2 (19, 21)	665	OR 4.17; 95% CI 0.63 to 27.58	0.14
Arrhythmia	2 (21, 22)	693	OR 1.44; 95% CI 0.87 to 2.39	0.16
QT prolongation	3 (19, 21, 22)	866	OR 1.8; 95% CI 0.79 to 4.11	0.16
Cardiac arrest	1 (21)	492	OR 2.17; 95% CI 1.16 to 4.07	0.02*
Diarrhea	2 (21, 28)	865	OR 0.8; 95% CI 0.34 to 1.85	0.60
Hypoglycemia	1 (21)	492	OR 1.23; 95% CI 0.43 to 3.51	0.70
Hydroxy-chloroquine (HCQ) and azithromycin (AZM) vs. control				
Escalation of respiratory support (including MV)	4 (19, 21, 25, 27)	2,294	OR 2.18; 95% CI 0.63 to 7.57	0.22
Development of severe disease	1 (21)	492	OR 3.19; 95% CI 2.07 to 4.91	<0.001*
Duration of hospitalization (d)	2 (21, 25)	1,180	MD 3.6; 95% CI 1.6 to 5.61	<0.001*
Adverse events				
Abnormal ECG	1 (21)	492	OR 2.28; 95% CI 1.51 to 3.44	<0.001*
QT prolongation	1 (21)	492	OR 1.98; 95% CI 1.08 to 3.63	0.03*
Arrhythmia	1 (21)	492	OR 2.21; 95% CI 1.38 to 3.52	<0.001*
Cardiac arrest	1 (21)	492	OR 2.52; 95% CI 1.44 to 4.42	0.001*
Diarrhea	1 (21)	492	OR 1.68; 95% CI 0.96 to 2.92	0.07
Hypoglycemia	1 (21)	492	OR 1.26; 95% CI 0.51 to 3.12	0.61
Hydroxy-chloroquine (HCQ) vs. HCQ and azithromycin (AZM)				
Escalation of respiratory support (including MV)	4 (19, 21, 25, 27)	1,730	OR 0.66; 95% CI 0.41 to 1.05	0.08
Development of severe disease	1 (21)	1,006	OR 0.53; 95% CI 0.38 to 0.75	<0.001*
Duration of hospitalization (d)	1 (25)	262	MD −1.0; 95% CI −2.46 to 0.46	0.18
Adverse events				
Abnormal ECG	1 (21)	1,006	OR 1.01; 95% CI 0.74 to 1.38	0.94
QT prolongation	2 (21, 22)	1,207	OR 1.28; 95% CI 0.88 to 1.87	0.20
Arrhythmia	2 (21, 22)	1,207	OR 0.74; 95% CI 0.52 to 1.06	0.10
Cardiac arrest	1 (21)	1,006	OR 0.86; 95% CI 0.58 to 1.29	0.46
Diarrhea	1 (21)	1,006	OR 0.68; 95% CI 0.41 to 1.1	0.12
Hypoglycemia	1 (21)	1,006	OR 0.98; 95% CI 0.45 to 2.12	0.95

OR, Odds ratio; MD, Mean difference; CI, Confidence interval; NE, Not estimable; PCR, Polymerase chain reaction; MV, Mechanical ventilation; ECG, Electrocardiogram.
**P < 0.05 significant.*

DISCUSSION

Summary of Evidence

After an extensive search of the literature, we included 17 studies with data of 8,071 participants. Compared to control, HCQ alone (not HCQ+AZM combination) has no significant effect on mortality or risk of adverse cardiac events. The evidence for all the outcomes was of "very low quality."

The high mortality and increased risk of adverse events with anti-malarial drugs noted by some studies may be overestimated because of the inclusion of an older population with underlying co-morbidities (including cardiac conditions) and simultaneous

TABLE 3 | GRADE evidence (anti-malarial drugs ± azithromycin vs. standard of care for patients with COVID-19 infection).

Outcomes	No of Participants (studies)	Quality of the evidence (GRADE)	Relative effect (95% CI)	Anticipated absolute effects	
				Risk with standard of care	Risk difference with anti-malarial drugs (95% CI)
Primary outcome measures (HCQ or HCQ+AZM vs. control/supportive care and high vs. low-dose CQ)					
All-cause mortality (HCQ vs. control)	5,944 (eight Observational studies)	⊕⊖⊖⊖ **VERY LOW**[a,b,c,d,e] due to risk of bias, inconsistency, indirectness, imprecision, publication bias	**OR 0.87** (0.46–1.64)	**Study population** **202 per 1,000**	**22 fewer per 1,000** (from 98 fewer to 91 more)
All-cause mortality (HCQ vs. control)	42 (one Non-RCT)	⊕⊖⊖⊖ **VERY LOW**[d,f,g,h,i,j] due to risk of bias, inconsistency, indirectness, imprecision	**OR 1.94** (0.07–50.57)	**Study population** **Not estimable**	**Not estimable ("0" event in control/standard of care group)**
All-cause mortality (HCQ+azithromycin vs. control)	2,310 (four Observational studies)	⊕⊖⊖⊖ **VERY LOW**[a,b,c,i] due to risk of bias, inconsistency, indirectness, publication bias	**OR 2.84 (2.19–3.69)**	**Study population** **94 per 1,000**	**133 more per 1,000** (from 91 more to 182 more)
All-cause mortality (HCQ vs. HCQ+azithromycin)	1,988 (five Observational studies)	⊕⊖⊖⊖ **VERY LOW**[a,c,d,f,h] due to risk of bias, inconsistency, indirectness, imprecision, publication bias	**OR 0.7** (0.54–0.9)	**Study population** **226 per 1,000**	**46 fewer per 1,000** (from 2 fewer to 83 fewer)
All-cause mortality (CQ: high-dose vs. low-dose)	81 (one RCT)	⊕⊖⊖⊖ **VERY LOW**[d,f,i,k] due to risk of bias, inconsistency, indirectness, imprecision	**OR 3.63** (1.24–10.58)	**Study population** **150 per 1,000**	**240 more per 1,000** (from 30 more to 501 more)
Secondary outcome measures (HCQ vs. control/supportive care)**					
Duration of hospitalization (day)	1,858 (five Observational studies)	⊕⊖⊖⊖ **VERY LOW**[a,c,e,f,h] due to risk of bias, inconsistency, indirectness, publication bias	**MD 2.17** (0.21–4.13)		The mean duration of hospitalization (day) in the intervention groups was **2.17 higher** (0.21 to 4.13 higher)
Any adverse events	264 (four RCTs)	⊕⊖⊖⊖ **VERY LOW**[c,f,g,h,j] due to risk of bias, inconsistency, indirectness, publication bias	**OR 3.35** (1.58–7.13)	**Study population** **145 per 1,000**	**217 more per 1,000** (from 66 more to 402 more)
Proportions with negative COVID-19 PCR after day 14	407 (two Observational studies)	⊕⊖⊖⊖ **VERY LOW**[a,c,f,h] due to risk of bias, indirectness, inconsistency, publication bias	**OR 6.37** (3.01–13.48)	**Study population** **594 per 1,000**	**309 more per 1,000** (from 221 more to 358 more)

The basis for the **assumed risk (e.g., the median control group risk across studies) is provided in footnotes. The **corresponding risk** (and its 95% confidence interval) is based on the assumed risk in the comparison group and the **relative effect** of the intervention (and its 95% CI). CI, Confidence interval; OR, Odds ratio; MD, Mean difference.*

***Secondary outcomes reporting pooled results from minimum two studies with significant difference between groups are reported here.*

*GRADE Working Group grades of evidence **High quality:** Further research is very unlikely to change our confidence in the estimate of effect. **Moderate quality:** Further research is likely to have an important impact on our confidence in the estimate of effect and may change the estimate. **Low quality:** Further research is very likely to have an important impact on our confidence in the estimate of effect and is likely to change the estimate. **Very low quality:** We are very uncertain about the estimate.*

[a] case-control study. [b] Inhomogeneous population with many being >65 years and male (not matched for age and sex confounders). [c] Patients in both the groups also received additional treatment which might influence the outcome, but not clearly defined. [d] The 95% CI around the pooled effect is wide and different in the included studies. The 95% CI includes no effect. [e] Being published on pre-print server and not in a peer-reviewed journal. [f] Both the groups were not homogenous considering the age and sex of the participants. [g] Open label trials. [h] Different dose schedule of intervention used. [i] Single study. [j] Single country data. [k] Though described as double-blinded, blinding of investigators, participants, and outcome accessor unclear. Allocation concealment also unclear. [k] One trial is open label. [l] Significant statistical heterogeneity. [m] Two trials are open label and one double-blinded (but this trial has unclear blinding and allocation concealment). [n] Wider 95% CI. [o] Developed country setting data that cannot be apply to developing country setting.

use of other cardiotoxic drugs (e.g., Azithromycin, and Oseltamivir). The same may be difficult to know during the current pandemic as there is no definitive treatment, and healthcare professionals all over the world want to administer these experimental drugs with the hope of saving some lives. The use of HCQ+AZM has drawn attention, and there are

differences in opinion regarding use of this combination. Compared to control, HCQ+AZM combination was found to increase the mortality rate significantly, in contrast to HCQ alone. Compared to HCQ+AZM combination, HCQ alone was significantly decreasing the mortality rate. These indirect evidences suggest that HCQ+AZM might increase the mortality rate, and caution should be exercised while using this combination in vulnerable population (e.g., those with advanced age, underlying cardiac conditions, and those receiving medication with cardiac side-effects, as noted in the included studies).

It has to be kept in mind that, the anti-viral action of anti-malarial drugs against COVID-19 is still largely unknown (49, 50). An acute systemic inflammatory reaction/cytokine storm (besides the viral infection itself) is the hallmark of COVID-19 infection (51). This reaction, once well-established, can cause rapid disease progression leading to death (52, 53). However, except for three studies (15, 23, 28), no studies have reported the effect of anti-malarial drugs on the inflammatory markers and blood counts (lymphocyte, neutrophil). As supportive treatments were not uniform across the included studies, one may argue that simultaneous use of other drugs (anti-viral drugs, and/or interferon-α) as a part of supportive treatment might have confounded (increased or decreased) the efficacy of the anti-malarial drugs (15). This possibility, however, seems less likely, as few studies have found no difference after excluding patients receiving these drugs (15).

An interesting observation was that, studies from USA showed a significantly increased risk of mortality compared to those from outside USA. The same could be explained by the following points in the USA study cohort: inclusion of a higher proportion of patients with severe or critical illness, advanced age, and co-morbidities. Among the included studies in the present review, marked variation (high heterogeneity) was noted in the age group (in the clinical trials majority were $\leq$50 yr of age, whereas, in the observational studies majority were $\geq$60 yr of age), severity of COVID-19 illness (around 72% of participants in the clinical trials were having mild and moderate illness, whereas <40% of the participants in the observational studies exhibited mild and moderate illness), and inclusion of patients with co-morbidities (diabetes, cardio-vascular disease, chronic lung disease, etc.) among the study cohorts. We could not, however, carry out sub-group analyses as per severity illness because of paucity of data. Except for the severity of COVID-19 illness, the remaining two characteristics (age group and inclusion of patients with co-morbidities) of the study cohort could increase mortality that is independent of the effect of CQ/HCQ (±Azithromycin). This emphasizes the role of randomized double-blind trials in establishing the actual efficacy (if any) of anti-malarial drugs, as the chance of selection bias would be very low, and the groups would be comparable. The dose schedule of CQ was nearly uniform; however, the dose schedule of HCQ varied widely among the studies (except for one large study, the cumulative dose was equal or higher than the recommended schedule in the remaining studies). There was, however, no difference in the mortality rate. The median time from onset of symptom to admission or treatment initiation was nearly $\leq$8 days in all but two studies, and, apart from one study, others used CQ/HCQ within 48 h of admission/hospitalization (not symptom onset). There was no significant difference in the mortality rate between exposure/interventions and controls in these sub-groups. This might be due to the fact that starting anti-viral drugs (including HCQ/CQ) after 48 h of symptom onset might not be beneficial as the golden window for antiviral treatment (e.g., in influenza) is lost (48). This is difficult in a hospitalized setting (may be possible in outpatient or community setting); however, one RCT could able to use it within 48 h of symptom onset (found a significantly shorter time to clinical recovery and pneumonia resolution without any mortality) (17).

Limitations

The studies were variable in many aspects (blinding of participants and outcome assessors, patient selection, severity of illness, dose schedule of the anti-malarial drugs, timing of administration, measurement of inflammatory markers and effect of the drugs on these markers, outcome definition, and measurements). We could not determine the effect of anti-malarial drugs in Covid-19 infection in pediatric and adolescent population. As there were few studies, results from all the secondary outcomes could not be pooled.

Future Areas of Research

Future clinical trials should include good quality RCTs with adequate sample size, should ideally be multi-centric, and should focus on the variability noted in the present review. Pediatric and adolescent population also need to be included in the ongoing studies to guide recommendation in this group of patients. Both CQ/HCQ should also be evaluated in non-hospitalized patients with COVID-19 infection.

CONCLUSIONS

As very low quality evidence suggests an increased risk of mortality and adverse event with HCQ plus Azithromycin combination (not HCQ alone); caution should be exercised while prescribing this combination for treatment of hospitalized adults with COVID-19 infection. Multi-centric RCTs (including both hospitalized and non-hospitalized patients) of a good quality are required for any firm recommendation to be made during the ongoing pandemic.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

RD developed the concept, abstracted data from papers, managed data for the review, performed the statistical analysis, interpreted data, made statistical inferences, wrote the review, and serves as

guarantor for the review. NisJ, ND, NikJ, and SN abstracted data from papers, managed data for the review, and wrote the review. JS developed the concept, performed the statistical analysis and made inferences, and wrote the review. RD and JS jointly act as guarantors. All the authors have approved the version to be published.

ACKNOWLEDGMENTS

The authors acknowledge ICMR center for advanced research in evidence based child health, PGIMER, and ICMR Headquarters.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2020.00482/full#supplementary-material

Supplementary Figure 1 | All-cause mortality (HCQ vs. control; Non-RCT).

Supplementary Figure 2 | All-cause mortality (High-dose vs. low-dose CQ; RCT).

Supplementary Figure 3 | Any adverse events (HCQ vs. control; RCTs).

Supplementary Figure 4 | Funnel plot (primary outcome data from observational studies).

REFERENCES

1. *WHO Coronavirus Disease (COVID-19) Dashboard*. Available online at: covid19.who.int (accessed July 9, 2020).
2. Zhang J, Xie B, Hashimoto K. Current status of potential therapeutic candidates for the COVID-19 crisis. *Brain Behav Immun*. (2020) 87:59–73. doi: 10.1016/j.bbi.2020.04.046
3. Vincent MJ, Bergeron E, Benjannet S, Erickson BR, Rollin PE, Ksiazek TG, et al. Chloroquine is a potent inhibitor of SARS coronavirus infection and spread. *Virol J*. (2005) 2:69. doi: 10.1186/1743-422X-2-69
4. Wan Y, Shang J, Graham R, Baric RS, Li F. Receptor recognition by the novel coronavirus from Wuhan: an analysis based on decade-long structural studies of SARS coronavirus. *J Virol*. (2020) 94:e00127–20. doi: 10.1128/JVI.00127-20
5. Zhou P, Yang X-L, Wang X-G, Hu B, Zhang L, Zhang W, et al. A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature*. (2020) 579:270–3. doi: 10.1038/s41586-020-2012-7
6. Devaux CA, Rolain J-M, Colson P, Raoult D. New insights on the antiviral effects of chloroquine against coronavirus: what to expect for COVID-19? *Int J Antimicrob Agents*. (2020) 55:105938. doi: 10.1016/j.ijantimicag.2020.105938
7. Golden EB, Cho HY, Hofman FM, Louie SG, Schönthal AH, Chen TC. Quinoline-based antimalarial drugs: a novel class of autophagy inhibitors. *Neurosurg Focus*. (2015) 38:E12. doi: 10.3171/2014.12.FOCUS14748
8. Gbinigie K, Frie K. Should chloroquine and hydroxychloroquine be used to treat COVID-19? A rapid review. *BJGP Open*. (2020) 4:20X101069. doi: 10.3399/bjgpopen20X101069
9. Yao X, Ye F, Zhang M, Cui C, Huang B, Niu P, et al. *In vitro* antiviral activity and projection of optimized dosing design of hydroxychloroquine for the treatment of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). *Clin Infect Dis*. (2020). doi: 10.1093/cid/ciaa237. [Epub ahead of print].
10. Sahraei Z, Shabani M, Shokouhi S, Saffaei A. Aminoquinolines against coronavirus disease 2019 (COVID-19): chloroquine or hydroxychloroquine. *Int J Antimicrob Agents*. (2020) 55:105945. doi: 10.1016/j.ijantimicag.2020.105945
11. Juurlink DN. Safety considerations with chloroquine, hydroxychloroquine and azithromycin in the management of SARS-CoV-2 infection. *CMAJ*. (2020) 192:E450–3. doi: 10.1503/cmaj.200528
12. Beaumont P, Ratcliffe R. Chloroquine: Trump's misleading claims spark hoarding and overdoses. *The Guardian* (2020 March 25). Available online at: https://www.theguardian.com/science/2020/mar/25/can-chloroquine-reallyhelp-treat-coronavirus-patients (accessed June 7, 2020).
13. Gautret P, Lagier JC, Parola P, Hoang VT, Meddeb L, Mailhe M, et al. Hydroxychloroquine and azithromycin as a treatment of COVID-19: results of an open-label non-randomized clinical trial. *Int J Antimicrob Agents*. (2020). doi: 10.1016/j.ijantimicag.2020.105949. [Epub ahead of print].
14. Chen J, Liu D, Liu L, Liu P, Xu Q, Xia L, et al. A pilot study of hydroxychloroquine in treatment of patients with moderate COVID-19. Zhejiang *Da Xue Xue Bao Yi Xue Ban*. (2020) 49:215–9. doi: 10.3785/j.issn.1008-9292.2020.03.03
15. Tang W, Cao Z, Han M, Wang Z, Chen J, Sun W, et al. Hydroxychloroquine in patients with mainly mild to moderate coronavirus disease 2019: open label, randomised controlled trial. *BMJ*. (2020) 369:m1849. doi: 10.1136/bmj.m1849
16. Huang M, Tang T, Pang P, Li M, Ma R, Lu J, et al. Treating COVID-19 with Chloroquine. *J Mol Cell Biol*. (2020) 12:322–5. doi: 10.1093/jmcb/mjaa014
17. Chen Z, Hu J, Zhang Z, Jiang S, Han S, Yan D, et al. Efficacy of hydroxychloroquine in patients with COVID-19: results of a randomized clinical trial. *medRxiv*. (2020). doi: 10.1101/2020.03.22.20040758. [Epub ahead of print].
18. Borba M, de Almeida Val F, Sampaio VS, Alexandre MAA, Melo GC, Brito M, et al. Effect of high vs low doses of chloroquine diphosphate as adjunctive therapy for patients hospitalized with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection: a randomized clinical trial. *JAMA Netw Open*. (2020) 3:e208857. doi: 10.1001/jamanetworkopen.2020.8857
19. Mahévas M, Tran VT, Roumier M, Chabrol A, Paule R, Guillaud C, et al. Clinical efficacy of hydroxychloroquine in patients with covid-19 pneumonia who require oxygen: observational comparative study using routine care data. *BMJ*. (2020) 369:m1844. doi: 10.1136/bmj.m1844
20. Geleris J, Sun Y, Platt J, Zucker J, Baldwin M, Hripcsak G, et al. Observational study of hydroxychloroquine in hospitalized patients with Covid-19. *N Engl J Med*. (2020) 382:2411–8. doi: 10.1056/NEJMoa2012410
21. Rosenberg ES, Dufort EM, Udo T, Wilberschied LA, Kumar J, Tesoriero J, et al. Association of treatment with hydroxychloroquine or azithromycin with in-hospital mortality in patients with COVID-19 in New York State. *JAMA*. (2020) 323:e208630. doi: 10.1001/jama.2020.8630
22. Saleh M, Gabriels J, Chang D, Kim BS, Mansoor A, Mahmood E, et al. The effect of chloroquine, hydroxychloroquine and azithromycin on the corrected QT interval in patients with SARS-CoV-2 infection. *Circ Arrhythm Electrophysiol*. (2020) 13:CIRCEP.120.008662. doi: 10.1161/CIRCEP.120.008662
23. Yu B, Li C, Chen P, Zhou N, Wang L, Li J, et al. Low dose of hydroxychloroquine reduces fatality of critically ill patients with COVID-19. *Sci China Life Sci*. (2020). doi: 10.1007/s11427-020-1751-3. [Epub ahead of print].
24. Huang M, Li M, Xiao F, Pang P, Liang J, Tang T, et al. Preliminary evidence from a multicenter prospective observational study of the safety and efficacy of chloroquine for the treatment of COVID-19. *Natl Sci Rev*. (2020). doi: 10.1101/2020.04.26.20081059. [Epub ahead of print].
25. Magagnoli J, Narendran S, Pereira F, Cummings TH, Hardin JW, Sutton SS, et al. Outcomes of hydroxychloroquine usage in United States veterans hospitalized with COVID-19. *Med*. (2020). doi: 10.1016/j.medj.2020.06.001. [Epub ahead of print].
26. Ayerbe L, Risco C, Ayis S. The association between treatment with heparin and survival in patients with Covid-19. *J Thromb Thrombolysis*. (2020) 50:1–4. doi: 10.1101/2020.05.27.20114694
27. Kuderer NM, Choueiri TK, Shah DP, Shyr Y, Rubinstein SM, Rivera DR, et al. Clinical impact of COVID-19 on patients with cancer (CCC19): a cohort study. *Lancet*. (2020) 395:1907–18. doi: 10.1016/S0140-6736(20)31187-9
28. Mallat J, Hamed F, Balkis M, Mohamed MA, Mooty M, Malik A, et al. Hydroxychloroquine is associated with slower viral clearance in clinical COVID-19 patients with mild to moderate disease: a retrospective study. *medRxiv [Preprint]*. (2020). doi: 10.1101/2020.04.27.20082180

29. Membrillo FJ, Ramírez-Olivencia G, Estébanez M, de Dios B, Herrero MD, Mata T, et al. Early hydroxychloroquine is associated with an increase of survival in COVID-19 patients: an observational study. *Preprints*. (2020). doi: 10.20944/preprints202005.0057.v2. [Epub ahead of print].
30. Chen X, Zhang Y, Zhu B, Zeng J, Hong W, He X, et al. Associations of clinical characteristics and antiviral drugs with viral RNA clearance in patients with COVID-19 in Guangzhou, China: a retrospective cohort study. *medRxiv [Preprint]*. (2020). doi: 10.1101/2020.04.09.20058941
31. Feng Z, Li J, Yao S, Yu Q, Zhou W, Mao X, et al. The use of adjuvant therapy in preventing progression to severe pneumonia in patients with coronavirus disease 2019: a multicenter data analysis. *medRxiv [Preprint]*. (2020). doi: 10.1101/2020.04.08.20057539
32. Shabrawishi MH, Naser AY, Alwafi H, Aldobyany AM, Touman AA. Negative nasopharyngeal SARS-CoV-2 PCR conversion in response to different therapeutic interventions. *medRxiv [Preprint]* (2020). doi: 10.1101/2020.05.08.20095679
33. Chowdhury MS, Rathod J, Gernsheimer J. A rapid systematic review of clinical trials utilizing chloroquine and hydroxychloroquine as a treatment for COVID-19. *Acad Emerg Med*. (2020) 27:493–504. doi: 10.1111/acem.14005
34. Sarma P, Kaur H, Kumar H, Mahendru D, Avti P, Bhattacharyya A, et al. Virological and clinical cure in COVID-19 patients treated with hydroxychloroquine: a systematic review and meta-analysis. *J Med Virol*. (2020) 92:776–85. doi: 10.1002/jmv.25898
35. *NIH Halts Clinical Trial of Hydroxychloroquine*. Available online at: https://www.nih.gov/news-events/news-releases/nih-halts-clinical-trial-hydroxychloroquine (accessed July 5, 2020).
36. Peng F, Tu L, Yang Y, Hu P, Wang R, Hu Q, et al. Management and treatment of COVID-19: the Chinese experience. *Can J Cardiol*. (2020) 36:915–30. doi: 10.1016/j.cjca.2020.04.010
37. *Management of Persons With COVID-19: National Institute of Health (NIH) Covid-19 Treatment Guidelines*. Available online at: https://www.covid19treatmentguidelines.nih.gov/overview/management-of-covid-19/ (accessed June 12, 2020).
38. Higgins JPT, Altman DG, Gøtzsche PC, Juni P, Moher D, Oxman AD, et al. The Cochrane Collaboration's tool for assessing risk of bias in randomised trials. *BMJ*. (2011) 343:d5928. doi: 10.1136/bmj.d5928
39. Wells G, Shea B, O'Connell J, Peterson J. *The Newcastle Ottawa Scale (NOS) for Assessing the Quality of Nonrandomised Studies in Meta-Analysis*. Available online at: http://www.ohri.ca/programs/clinical_epidemiology/oxford.asp (accessed June 12, 2020).
40. Sterne JA, Hernán MA, Reeves BC, Savovic J, Berkman ND, Viswanathan M, et al. ROBINS-I: a tool for assessing risk of bias in non-randomised studies of interventions. *BMJ*. (2016) 355:i4919. doi: 10.1136/bmj.i4919
41. *Review Manager (RevMan) [Computer program]. Version 5.3*. Copenhagen; London, UK: The Nordic Cochrane Center; The Cochrane Collaboration (2014).
42. Luo D, Wan X, Liu J, Tong T. Optimally estimating the sample mean from the sample size, median, mid-range, and/or mid-quartile range. *Stat Methods Med Res*. (2018) 27:1785–805. doi: 10.1177/0962280216669183
43. Schmidt FL, Oh IS, Hayes TL. Fixed- versus random-effects models in meta-analysis: model properties and an empirical comparison of differences in results. *Br J Math Stat Psychol*. (2009) 62:97–128. doi: 10.1348/000711007X255327
44. Higgins JPT, Green S. *Cochrane Handbook for Systematic Reviews of Interventions Version 5.0.0. The Cochrane Collaboration*. (2008). Available online at: www.cochrane-handbook.org (accessed June 12, 2020).
45. Sterne JA, Egger M, Smith GD. Systematic reviews in health care: investigating and dealing with publication and other biases in meta-analysis. *BMJ*. (2001) 323:101–5. doi: 10.1136/bmj.323.7304.101
46. *GRADEpro GDT: GRADEpro Guideline Development Tool [Software]*. McMaster University (developed by Evidence Prime, Inc.) (2015). Available online at: gradepro.org (accessed June 12, 2020).
47. Schünemann H, Brozek J, Guyatt G, Oxman A, editors. GRADE handbook for grading quality of evidence and strength of recommendations. The GRADE Working Group (2013). Available online at: guidelinedevelopment.org/handbook (accessed June 12, 2020).
48. Harper SA, Bradley JS, Englund JA, File TM, Gravenstein S, Hayden FG, et al. Expert Panel of the Infectious Diseases Society of America. Seasonal influenza in adults and children–diagnosis, treatment, chemoprophylaxis, and institutional outbreak management: clinical practice guidelines of the Infectious Diseases Society of America. *Clin Infect Dis*. (2009) 48:1003–32. doi: 10.1086/598513
49. Touret F, de Lamballerie X. Of chloroquine and COVID-19. *Antiviral Res*. (2020) 177:104762. doi: 10.1016/j.antiviral.2020.104762
50. Barnard DL, Day CW, Bailey K, Heiner M, Montgomery R, Lauridsen L, et al. Evaluation of immunomodulators, interferons and known *in vitro* SARS-coV inhibitors for inhibition of SARS-coV replication in BALB/c mice. *Antivir Chem Chemother*. (2006) 17:275–84. doi: 10.1177/095632020601700505
51. Guan WJ, Ni ZY, Hu Y, Liang W-H, Ou C-Q, He J-X, et al. Clinical characteristics of coronavirus disease 2019 in China. *N Engl J Med*. (2020) 382:1708–20. doi: 10.1056/NEJMoa2002032
52. Chen T, Wu D, Chen H, Yan W, Yang D, Chen G, et al. Clinical characteristics of 113 deceased patients with coronavirus disease 2019: retrospective study. *BMJ*. (2020) 368:m1091. doi: 10.1136/bmj.m1091
53. Zhou F, Yu T, Du R, Fan G, Liu Y, Liu Z, et al. Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: a retrospective cohort study. *Lancet*. (2020) 395:1038, 1054–62. doi: 10.1016/S0140-6736(20)30566-3

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Communication

ORIGINAL RESEARCH
published: 29 July 2020
doi: 10.3389/fcomm.2020.00062

Culture-Centered Processes of Community Organizing in COVID-19 Response: Notes From Kerala and Aotearoa New Zealand

Mohan J. Dutta, Christine Elers and Pooja Jayan*

School of Communication, Journalism and Marketing, Center for Culture-Centered Approach to Research and Evaluation (CARE), Massey University, Palmerston North, New Zealand

The culture-centered approach (CCA) foregrounds the organizing role of communities at the "margins of the margins" of the globe as the spaces for identifying the structural challenges to health and well-being and for co-creating community-anchored solutions to these challenges. Pandemics such as COVID-19 render visible the deep-rooted inequalities across and within societies, seeded and catalyzed by over three decades of variegated neoliberal reforms. The trajectories of COVID-19 outbreaks as well as the effects of COVID-19-related policies render visible the inequalities that are written into the neoliberal organizing of political economy. Community participation is scripted into the neoliberal framework as an instrument for depoliticizing community and utilizing it as a channel for disseminating top-down individual behavior change messages. Drawing on the examples of community organizing in Kerala where the Communist Party of India (Marxist) has actively co-created an infrastructure for socialist organizing, and Iwi-led Maori checkpoints in Aotearoa New Zealand, we delineate the features of transformative community organizing. Community organizing in the CCA is political, foregrounding community sovereignty as the basis for resisting neoliberal health structures. Community struggles for communication equality thus point to alternative forms of organizing health and well-being that challenge and seek to dismantle neoliberal governmentality.

Keywords: COVID-19, culture—centered communication, community organizing, Maori organizing, Kerala, socialist organizing, Labor, Communist Party of India (Marxist)

OPEN ACCESS

Edited by:
Iccha Basnyat, James Madison University, United States

Reviewed by:
Satarupa Dasgupta, Ramapo College, United States
Shamshad Khan, University of Texas at San Antonio, United States

****Correspondence:***
Mohan J. Dutta
m.j.dutta@massey.ac.nz

Specialty section:
This article was submitted to Health Communication, a section of the journal Frontiers in Communication

Received: *12 May 2020*
Accepted: *13 July 2020*
Published: *29 July 2020*

Citation:
Dutta MJ, Elers C and Jayan P (2020) Culture-Centered Processes of Community Organizing in COVID-19 Response: Notes From Kerala and Aotearoa New Zealand. Front. Commun. 5:62. doi: 10.3389/fcomm.2020.00062

The culture-centered approach (CCA) foregrounds the organizing role of communities[1] at the "margins of the margins"[2] (Dutta, 2020a) of the globe as the spaces for identifying the structural challenges to health and well-being and for co-creating community-anchored solutions to these challenges. Pandemics such

[1]The culture-centered approach (CCA) constructs communities as contested, dynamic, and unequal terrains, constituted in relationships of power. Communities are sites of interrogating power as well as creating radical equality through struggles for power. Interrogating the hegemonic notion of the community as monolith, the CCA suggests that communities are strategically constructed, crafting specific identities directed toward achieving certain goals. In culture-centered co-constructions with the margins, communities are depicted as spaces at the margins, forged through communicative processes to articulate demands.

[2]The concept of the "margins of the margins" reflects the processes of discursive erasure and material marginalization constituted in community organizing spaces in marginalized contexts. As a theoretical, methodological and practical anchor, the "margins of the margins" point toward the vitality of collective reflexivity as the basis for continually attending to erasure and the necessity for building communicative equality that is open-ended.

as COVID-19 render visible the deep-rooted inequalities across and within societies (Dutta, 2016, 2020b, 2020e; Ahmed et al., 2020; Van Lancker and Parolin, 2020), seeded and catalyzed by over three decades of variegated neoliberal reforms (Brenner et al., 2010). The trajectories of COVID-19 outbreaks as well as the expulsions and displacements produced by COVID-19 policy responses across states foreground the urgent necessity of structurally directed social change communication amid the pandemic. As witnessed across the globe, COVID-19 is reproducing, catalyzing, and circulating existing inequalities that have been produced by increasingly extreme neoliberal reforms, further exacerbating the already disenfranchising conditions experienced by large proportions of those at the "margins of the margins" (Dutta, 2020a). The process of cultural centering anchors communicative responses to pandemics in community voices, constituted in the work of everyday organizing for radical democracy in communities at the margins to transform the unhealthy structures and generate socialist state responses owned by and accountable to communities. The spaces for community voices at the margins make visible the violence entrenched into market-promoting neoliberal reforms, and the powerful effects of these reforms on human health and well-being; simultaneously, these community spaces re-organize local-regional-national-global linkages in the rationality of communicative, political, and economic equality (Dutta, 2016).

Meaningful responses to pandemics are constituted in communities, situated amidst grassroots democratic decision-making and community negotiations of the structural contexts of the pandemic. The organizing role of communication draws on the agentic capacities of communities in identifying and responding to challenges through the ongoing work of building dialogic infrastructures for community voices. Communication is constitutive of community, and mediates community action through an iteratively reflexive process of interrogating power (Dutta and de Souza, 2008). It forms the infrastructure, fabric, and texture of community life and is in turn, constituted by community. Drawing on the extant scholarship on the linkages between communication and community in the CCA, we theorize communication as the organizing space for co-creating responses to the pandemic. As a resource embedded in everyday community life, communication brings together people in spaces, creating the basis of shared values, shared meanings, and shared actions. These shared values, meanings, and actions form the basis of local, national, and global responses to pandemics. We argue that democratic community action in pandemic/crisis response is strengthened when state structure support socialist economic organizing, guaranteeing the fundamental resources of health as a human right.

In its role as a dialogic anchor for creating connections, communication brings the "margins of the margins" in collectives, and organizes and sustains relationships that form communities. The manuscript draws on two case studies of emergent success in responding to COVID-19, one from the Indian state of Kerala and the other from the context of Maori organizing in Aotearoa to depict the organizing roles of communities in interplay with the organizing structure of the state in responding to COVID-19. Both the state of Kerala under the Communist Party of Indian (Marxist) and Aotearoa New Zealand under Labor reflect a certain level of commitment to socialist political-economic organizing, although this is negotiated amidst turns toward neoliberal policies internally as well as within a broader global climate of pressures exerted by the transnational capitalist class and international financial institutions (IFIs) for further neoliberal reforms. In the case of Kerala, the response of the democratically elected Communist state is situated within a broader anti-science neo-fascist right-wing federal government further pushing extreme neoliberalism within an already neoliberal structure (Cammaerts, 2020). Through this comparative work that delves into local, national, regional, and global responses, we examine closely the role of community organizing rooted in radical democracy in relationship with processes of socialist state organizing in constituting effective pandemic response. Our analysis of responses and comparison of community responses foregrounds the following key threads in community organizing in response to COVID-19: (a) addressing structures, (b) mobilizing resources, (c) drawing on cultural values, (d) anchoring in social justice, and (e) fostering spaces for democratic ownership.

Drawing on the key tenets of the CCA, we explore community organizing that co-creates communication infrastructures for imagining, creating, and sustaining socially just responses to the pandemic at the global margins. The communication infrastructures work toward fostering communicative equality through the democratization of spaces of decision-making, while simultaneously negotiating continually a politics of structural transformation based on an ongoing commitment to socialist principles for organizing health, education, housing, food, and work (Dutta, 2008, 2011, 2016; Dutta and de Souza, 2008). Organizing processes that foster socialist radical democracies at the global margins address both the trajectories of spread of the virus as well as the overarching structural inequalities that constitute deep unequal effects of pandemic response policies. Culture-centered health communication interventions recognize the material inequalities that constitute health and well-being, thus imagining broader transformations in economic structures, with fundamental provisions of universal basic income, universal housing, universal food, and universal health. The two cases offered for comparison in this manuscript will empirically examine the forms of communicative response anchored in culture-centered processes that seek transformations in political, social, and economic organizing[3]. In resistance to the hegemonic framework of health communication that keeps neoliberal structures intact through individualizing responses, communities at the margins as spaces of democratic response offer imaginaries for organizing health and well-being that are not only responsive

[3]In constructing the cases, we will draw from published news reports, reports by civil society organizations, as well as academic reports.

to COVID-19 in the short term, but also potent in their transformative capacities for re-organizing political economies (Habersaat et al., 2020).

CULTURE-CENTERED APPROACH AND PANDEMIC COMMUNICATION

Working with the question of voice, the CCA examines the sites of erasure in hegemonic formations, the various layers at which erasures are codified into these structures, and the ways in which voices are erased from spaces of decision-making (Dutta, 2005, 2007, 2018, 2019). These erasures of voices, especially voices from communities at the margins, are situated amidst an overarching ideology of neoliberalism that positions health problems as problems of individual behavior, to be targeted through expertise-driven top-down models of health communication. The locus of decision-making is driven by experts, with elite technocratic managerialism driving health communication. The behaviors recommended as well as the communication surrounding the recommended behaviors are located in the ambits of expertise. Very much embedded within the logics of the neoliberal status quo, models of pandemic communication problematize the pandemic as resulting from the behaviors of individuals. Pandemic communication in the hegemonic framework targets individual attitudes, knowledge, and behavior, then proposing messaging strategies directed at changing these individual behaviors in order to stop the spread of the pandemic.

Entire industries of behavior change communication have been put forth within the hegemonic logic of individualized health communication, while simultaneously backgrounding the structural inequalities that form the fabrics of health problems. In the context of pandemics, the structural violence that constitutes the trajectories of spread of the pandemic as well as the precarities that are reproduced by the pandemic are rendered invisible while communities at the margins are turned into targets of top-down expert-led interventions. The problematization of health in the realm of individual behavior thus keeps the neoliberal structures intact, circulating erasures of the margins (Dutta, 2018, 2019). Resisting this neoliberal model of privatized individualism, the CCA centers the interplays of culture and community in health communication (Dutta, 2007, 2008; Dutta and Basu, 2008). In the context of pandemic communication, the foregrounding of community voices at the margins interrogates the hegemonic narratives of pandemic response, building structurally directed advocacy and activist interventions. The behavior change framework constituting the ideology of pandemic response globally is interrogated through the presence of subaltern voices that have hitherto been erased (Dutta, 2004).

Community Participation

Community participation serves as a register for bringing about change that is meaningful to the community (Dutta, 2018). The participation of the community is essential for comprehending the problems as experienced by community members, and creating solutions that are meaningful to their lived experiences (Dutta, 2004). Through evidence-based knowledge grounded in cultural meanings and everyday understandings embedded in community life, communities emerge as anchors to developing health communication solutions. In contrast to the concept of community participation serving as a conduit for diffusing the neoliberal agendas of hegemonic health communication solutions, community participation in the CCA is re-organized in the logics of organizing at the margins anchored in an active politics of resistance. The impetus of community participatory processes in the CCA emerges from within the margins as the basis of expressing individual, familial, and collective agency. The process of cultural centering re-works culture as an infrastructure of drawing on community values, community norms and community narratives to put forth health solutions that challenge the hegemonic structures and the capitalist/racist logics that constitute these structures.

Community Participation, Co-optation, and Neoliberalism

Community participation in the neoliberal ideology constitutes community as a depoliticized space for disseminating the top-down interventions designed by technocratic elites. Community-based participatory interventions, funded by neoliberal organizations such as the World Bank, International Monetary Fund, development agencies (USAID) and global foundations (Gates, Ford) organize communities as spaces for self-help, while simultaneously catalyzing the depletion of public resources and infrastructures (Dutta, 2013). Community voice, incorporated as community participation in hegemonic health interventions, serves the agendas of neoliberal expansion, incorporated into the ideology of growth as development. Participatory agency of communities at the margins is coded into self-help programs of community engagement that reproduce the ideology of the global free market, with individualized health solutions disseminated through community participatory infrastructures (Dutta, 2017). Participation serves to perpetuate the neoliberal ideology, constructing it in culturalist language, and developing culturally sensitive health communication solutions that perpetuate the neoliberal status quo. Participation maps and domesticates dissent, depoliticizing communities and incorporating subjects as engaged stakeholders to consolidate state-capitalist power configured in technocratic logics. Participation is an instrument for control and disciplined management built on the idea that voters are irrational, ignorant, and incapable of making informed decisions.

Community Participation and Resistance

The CCA positions community participation in resistance to this co-optive nature of participation in the service of global capital. Noting that the hegemonic forms of participation established within the ambits of the WB or global Foundations serve the expansionary logics of global capital, culture-centered interventions root themselves in the actual lived politics of co-creating communicative infrastructures for democracy at the global margins. Health is theorized amidst the participation of those at the global margins in

processes of resisting hegemonic structures, foregrounding local meanings, and working with these meanings to mobilize for change. The work of community participation in the CCA is centered on building spaces community democracy, centering community voice infrastructures in the participation of the "margins of the margins." The recognition that communities are not homogenous guides the co-creation of community infrastructures for the voices of the "margins of the margins." The framework for who participates and what the rules of participation are therefore emerges from within community spaces, guided by the conceptual anchors, "whose voices are missing from this discursive space," and "how can we co-create communicative spaces that seek out those at the margins."

Noting that voices from the margins reflect local agency, culture-centered processes attend to the agentic community ownership of the local organizing frameworks, which serve as the basis of securing health and well-being (Dutta, 2007, 2011, 2015, 2018; Basu and Dutta, 2009). Entrenched communicative inequalities are addressed through the co-creation of communicative processes of building spaces of local participation, embedded in local ownership over democratic processes of decision-making (Dutta et al., 2019), social change emerges from the imaginations of subaltern communities in the Global South. The CCA centers meanings as the basis of knowledge production, working with meanings to build infrastructures for securing resources, transforming politics, and co-creating infrastructures at the margins. These locally situated meanings therefore serve as the basis of theorizing communication for health and well-being. In the context of COVID-19, meanings of everyday health and wellbeing guide the organizing of communities, movements, and political parties.

The CCA suggests that de-centering the top-down framework of COVID-19 response calls for exploring expressions of collective agency at various levels and in various forms, including in communities, in workplaces, in worker unions, in civil societies, and in policy infrastructures that are critical to creating and sustaining responses anchored in care and justice. The pandemic constitutes the backdrop for the emergence of communicative leadership across spaces from communities to nation states to global organizations. In contrast, the absence of communicative leadership strains existing resources, seeds divisiveness, further fosters disinformation, and exacerbates the struggles experienced by the margins of highly unequal societies. COVID-19 makes visible the deep inequalities in contemporary political and economic organizing; it is in this backdrop that communities emerge as spaces for re-organizing meanings, conceptual anchors, and political economic systems.

HEGEMONIC COVID-19 RESPONSES

Hegemonic COVID-19 responses, situated within the framework of top-down, expertise-driven behavior change interventions constructs the spread of COVID-19 amidst individual-level behavior. The behavior change strategies and recommendations, formulated by dominant state actors, are framed as individual behaviors. The framing of behaviors in the realm of the individual perpetuates the neoliberal status quo, leaving intact the logics of "flow," "free market," "global movements," and "privatization of resources" that have led to the deep inequalities evident amidst COVID-19.

COVID-19 and the New Zealand Government's Response

As the global COVID-19 pandemic surfaced in Aotearoa, New Zealand, Prime Minister, Jacinda Ardern, announced on 23 March 2020 that New Zealand would move into lockdown level four at 11:59 p.m. on Wednesday 25 March 2020. On the announcement date, there were 102 positive cases of COVID-19 in New Zealand. The majority of cases were linked to overseas travel and two cases were treated as community transmission (Ministry of Health, 2020, Strongman, 2020). Sobering images of strained public healthcare systems and the rapid escalation of COVID-19 cases causing death from Italy and other infected areas of the world circulated social and national media prompting New Zealand's decisive elimination strategy to "go hard and go early" (Ardern, 2020 as cited in Hickey, 2020). It could be argued that global shortages of valuable ventilators to treat severely ill patients also influenced the government's strategy that closed all borders and locked down the country. A public health stock audit revealed that there were 520 ventilators in the public healthcare system (Checkpoint, 2020) with possible access to another 250 ventilators used by private hospitals and other organizations (Pennington, 2020). Should the hospitals become inundated with COVID-19 patients, the current stock would not be sufficient to artificially ventilate critical patients. Drawn from Bentham's version of utilitarianism (Gustafsson, 2018) where simply put, acts are justified and should be pursued if they produce the most good for the most people in society, the Italian College of Anesthesia, Analgesia, Resuscitation and Intensive Care published guidelines for doctors working in the intensive care units. These guidelines advised that age and comorbidity rates of infected patients should be considered during the triaging process to maximize the best use of healthcare facilities and equipment (Mounk, 2020; Ovadia, 2020). This reasoning means that the provision of healthcare, a basic humanitarian service, should be deployed for people who present with a greater success of recovery. Should positive COVID-19 cases exponentially rise in New Zealand and the public healthcare system becomes overwhelmed to the point where there are not enough healthcare facilities and equipment for everyone in need, then who decides who will be connected to a potentially life-saving ventilator and who will miss out? In other words, who decides who will live and who will die?

Historical Precedents

Against the backdrop of New Zealand's colonial settler state, the public healthcare system has failed Māori resulting in over-representation in many debilitating health statistics (Reid et al., 2019; Waitangi, 2019). These health outcome inequalities existed pre-COVID-19. Ngata (2020) highlights that the COVID-19 pandemic has not plunged the country into unprecedented times because like other nations, New Zealand has a historical litany of deadly pandemics that has plagued these shores (Day, 1999;

Chapple, 2016, 2018). The Spanish flu' 1918 pandemic struck deadly rates at 48.9 per 1,000 for Māori, massively higher than the *Pākehā* (European New Zealanders) morbidity rate of 6 per 1,000 (Rice, 2018).

Day (1999) provides a historical account of the 1913 smallpox epidemic and its prevalence amongst Māori communities. Furthermore, Day explains that the vaccine lymph thought to provide immunization against smallpox was produced for distribution to Māori, not out of kind benevolence but an attempt to curb further infection of Pākehā. Despite the Health department's publicized intent to distribute smallpox vaccines to Māori communities, Day added that most of the doses were swallowed up in urban areas by Pākehā, resulting in a shortfall with some rural northern Māori communities entirely missing out. Māori were prevented from traveling unless they carried a successful immunization certificate and even then, many were turned back on their journey and barred from entering towns and shops. Although Day notes the discriminating attitudes of Pākehā, her analysis falls short of naming the deep-seated racism that often accompanies pandemics, underpinned by the colonial servitude to Whiteness.

Hegemonic Response in India

It takes a pandemic to render visible the deep inequalities that make up the highly unequal societies we inhabit. As pandemics go, the power of COVID-19 lies in its mobility, along the circuits of global capital, picked up and carried by the upwardly mobile classes feeding the financial and technology hubs of capital. The irony of neoliberal globalization lies in the disproportionate burden of accelerated mobilities borne by the bodies of the poor at the global margins. The poor, whose bodies are the sites of neoliberal extraction, are also the bodies to be easily discarded when crises hit. The images of throngs of people, the poor, now expelled from their spaces of precarious work at the metropolitan centers of financial and technology capital in India, spaces that are projected as the poster-models of mobility in development propaganda, walking on the long walk home, are circulating across our mobile screens (Dutta, 2020c,d). Images of a migrant worker dead after the grueling walk home, a mother pulling her daughter as they try to make their way home, a young man bursting into tears at the sight of food, a father walking as he carries his sleeping daughter on his shoulders, crowds of workers waiting in long lines to board buses, these are the faces of the unequal India made visible by COVID-19. These images of emaciated men and women, with little children, carrying pots, torn down bags and dilapidated beddings on their heads, walking on the roads and highways that form the infrastructures of the new India are haunting reminders of the masses of displaced people expelled by wars, riots, genocides, and famines. These forced mobilities as expulsions reflect the worst excesses of neoliberal India, rife with caste-class hierarchies.

Deep Inequalities and Indian Society

Note in the backdrop of these images the high-rises and the gated communities that house India's upwardly mobile classes, the classes that fuel its financial and technological imaginaries. These are the classes that extract the daily labor of the precarious workers. Ironically, also these same classes are quick to catalyze the expulsion of precarious workers when they are turned into threats, by an inversion of empirical evidence (where the actual threats of the infection are largely carried by the upwardly mobile bodies traveling across global borders) and driven by irrational fears.

Authoritarian Repression and State Control

What is the most striking feature of the Indian lockdown is the paradox inherent in the state's management of COVID-19 (Roy, 2020). Even as the state has decreed Indians to stay indoors, replete with police violence targeting anyone that is seen outside, large crowds of migrant workers are on the streets, walking insurmountable distance to get home. Contrary to the 24/7 propaganda of the strong leader, the state here is weak and ineffective, demonstrating its lack of preparedness in addressing the needs of those at the margins. The poor governance, lack of preparedness, and mismanagement of the crisis are publicly on display, contrary to the propaganda the regime concocts regularly. The absence of careful planning and consideration of the needs of the migrant workers is evident in the absence of infrastructures of care. For instance, transportation facilities following precautionary measures are entirely missing. Similarly, transit-housing arrangements for precarious migrant workers following precautionary measures are entirely missing. Infrastructures for addressing everyday food needs of migrant workers are entirely absent.

All this is ironic in the backdrop of the power asserted by the authoritarian state to mobilize material resources quickly to set up infrastructures for marking and incarcerating so-called illegals picked up by its neo-fascist National Register of Citizens. The precarious workers that hold up the IT-finance economies in the metropole are discardable. Their bodies can be thrown off, disciplined, and violently targeted by the repressive state in its performance of governance. What's more, as amply evident on our screens, their bodies can be subjected to brutal violence and repression unleashed by the police as instruments of the state. As all this happens, the yuppies from the gated communities that benefit from the labor of these precarious bodies are all too comfortable that the threat has been managed and mitigated. Note the inversion at work here, much like other discourses of inversions carried out by the neoliberal regime. The burden of COVID-19, a virus carried into India by the chains of neoliberal mobility, has to be borne by India's underclasses. The performance of risk is an inversion of the actual sources of risk.

Depletion of Public Health

The poverty and precarity made visible by COVID-19 is constituted amidst extreme neoliberal reforms pursued by a state aligned with the interests of capital (Dutta, 2016). From the privatization of the telecommunication infrastructures to the privatization of the railways, the current regime is soaked in the worst excesses of the neoliberal ideology. This translates into the large-scale absence of financial infrastructures, welfare resources, food resources, and essential shelter infrastructures to address the needs of those in poverty. For a virus that thrives on mobility, guaranteeing these essential infrastructures

is central to managing the epidemic. With the Congress-led neoliberal reforms introduced in the 1990s to the BJP-led accelerated privatization of the Indian economy, the public health infrastructure in India has rapidly dwindled. The systematic attack on the public health infrastructure has been catalyzed by transnational Foundations such as the Bill and Melinda Gates Foundation, which in the name of addressing the HIV/AIDS epidemic, strategically invested into setting up a privatized management model. The hegemony of the mantra of public-private partnerships categorically dismantled the already minimal public health infrastructures.

COMMUNITY ORGANIZING AT THE MARGINS

In the backdrop of the larger structures of neoliberal organizing of COVID-19 response, both in Kerala and Aotearoa, grassroots community participation in dialogic conversation with a socialist state structure points toward other imaginaries, offering transformative registers for COVID-19 response. These imaginaries are explicitly grounded in community collective agency, depicting the resistive spaces of community life organized in principles of collective care. Aligned with the notion of the "pandemic as portal" (Roy, 2020), the empirically grounded practices of community organizing are constituted in relationship with the structures of the state, both in resistance to the structures of the state and in confluence with state formations. The ongoing negotiations of community response with state structures, practices and policies points to the tensile spaces of power constituted in the relationship between the state and community organizing. Organizing at the community is constituted by the state formation, and simultaneously organizes the state formation. The state structure in both Aotearoa New Zealand and Kerala is a site of ongoing negotiations of power between socialist commitments and neoliberal seductions. Whereas, on one hand, both states exist within a broader neoliberal climate that works by individualizing health care, on the other hand, the party politics in state formation in both Kerala and New Zealand articulates an explicitly socialist commitment to differing degrees.

A People-Centric Route to Rebuild the World From the Global South

One by one
Companies close down.
Life comes to a standstill
Everywhere in the land.
Lords are hardly bothered
Lords of the government are hardly bothered (Andalattu, 1987, as cited in Mannathukkaren, 2013).

As the world grapples with the COVID-19 pandemic these lines written decades ago by Andalattu, who often wrote about communism in Kerala, is of much significance. As Andalattu's poignant lines reverberated, life has come to a standstill now, and the lords are hardly bothered. The policy responses of some countries to COVID-19 reveal the paramount importance given to fiscal repercussions overlooking the health of its people. The reaction of states to thousands of affected people, dead and people in quarantine lay bare the model that has steered public policies over the last decades: the neoliberal model. COVID-19 has shown us a mirror to our society that the neo-liberal strategies have failed humanity. The divide in life chances between rich and poor is apparent, as poor populations lacking access to health services in standard settings are left most vulnerable during times of the COVID-19 debacle (Ahmed et al., 2020). The cracks in developed nations like the US and the UK are evident as the countries are bracing an unexpected peak in COVID-19 fatalities. Years of austerity and cutbacks in public health care have ruined the healthcare system of many such countries (Malliori et al., 2013; Stuckler et al., 2017). As a by-product of neoliberal procedures across public sector establishments, health care system encounters severe limitations at regular times like understaffing, over workload, low salaries, inadequate working conditions (Selberg, 2013) and hence are not likely to provide the desirable care in a crisis like COVID-19. The pursuit of wealth under the neo-liberal agendas has worsened the COVID-19 crisis and nations ability to tackle it. However, the small state of Kerala in India has shown to the world how putting human lives ahead of profit is critical.

Kerala had dealt with setbacks back to back, from severe floods in 2018 and 2019 to Nipah outbreak, a disease known to have no vaccine developed so far. In India, the first case of COVID-19 was reported in Kerala on January 30th, 2020, when a student who came to Kerala from Wuhan, China contracted the virus (Raghunath, 2020). Kerala with 35.1 million population is a much-touted tourist destination for international travelers and it has a massive number of expatriates who travels back and forth. These posed a risk for transmission among its population. Kerala's COVID-19 cases escalated in March first week when people started coming back from middle-eastern countries and Europe. At one stage, the state had the maximum number of contagions in India. As of May 9th, 2020, 503 cases were reported in Kerala, and four people died, amounting to $<1\%$ of the total reported cases. Whereas, India, in total, has 59,662 cases with 1,981 deaths (PTI, 2020). Kerala was able to flatten the curve because of its stable grass-root democracy and robust healthcare system (Biswas, 2020). The Guardian cites Kerala as an exemplar of COVID-19 response (Kurian, 2020). A Washington Post story states that aggressive testing and contact tracing is key to communist-run Kerala's triumph in tackling COVID-19 (Masih, 2020).

Socialist Organizing

In this section, we argue that Kerala's COVID-19 success is an offshoot of years of investment in healthcare and its people-centered development strategy. Kerala tops the human development index, compared to other states in India, and in sharp contrast to the state of Gujarat that is sold as the model of neoliberal growth and development (Singh, 2020). The state has high literacy rates and better health outcomes than other Indian states, with strong state support for education (Tripathi, 2019). Over the years, the Communist Party of India -Marxist [hereafter cited as CPI(M)]-led government has tremendously invested in education and in the healthcare system (Namboodiripad,

1984, 1994; Thomas and Jayesh, 2019; Raj, 2020) to assist the underprivileged sections of the society (Chasin and Franke, 1991). Kerala is one of the few states in India where left parties play an active role in the socio-political environment [other than the state of West Bengal where the CPI(M) has been on a decline because of problems with organizational structure as well as the targeted attacks organized by right-wing reactionary forces]. From the very outset of the first communist ministry in 1957, the left has played an inevitable role in shaping the public discourse and consciousness in Kerala (Singh, 2011). In 1979, a coalition called the Left Democratic Front was created, of which the CPI(M) is the largest political party (Bhatt, 2005). CPI(M) paved a new road to socialist democracy, where the party gave precedence to democratic institutions, practices, and policies in Kerala (Williams, 2017). Despite Kerala's precarious financial position, state governments of Kerala have never reversed any public service schemes, thus reiterating the commitment of state governments to the social sector (Heller and Isaac, 2005). The rhetoric of the Communist Party is instilled with strong overtones of well-being and CPI (M)'s election manifesto commits to the right to free health care. Under the current communist-led government, an initiative called "Aardram" was launched in Kerala to make public health care system more people-centric and to improve the amenities of public health facilities (Chetterje, 2020), thus slashing the dependence of public on private health facilities. The people centered policy by the left front and the grassroot participation carved a remarkable public health infrastructure for Kerala.

The communist state in Kerala, embedded within a larger democratic electoral politics, is embossed by substantial social expenditures and schemes targeted at the margins of society. Contrary to India's tryst with neoliberalism, the communist government in Kerala battled the neo-liberal policies encouraged by the Central government (Nair, 2007). Socio-political organizations in Kerala regularly submitted requests to officials for better health and educational facilities (Nag, 1983). Failure to meet public demands caused agitations, in some instance officers were *gheraoed* or encircled by agitators who did not permit them to leave the office premises until their demands were met (Franke and Chasin, 1992). In the 1970s, CPI(M) employed one of the most radical set of land reforms in the world to safeguard the rights of the tenants on land (Franke and Chasin, 1992). The communist government in 1996 took the agenda of decentralization as the priority and introduced the "People's Campaign for Decentralized Planning" as a commitment to democratic politics and to amplify the participation of community in planning process (Heller et al., 2007). The solidarity of communist government for class-based movements in Kerala (Heller, 2000) augmented social trust and paved way for many programmes with community participation. Under the communist regime an ascendance of neoliberalism is evident in Kerala, with high rates of unionization (Dreze and Sen, 2002; Thanickan, 2006). Participatory institutions and grass roots democracy under CPI(M) developed a unique state-civil society synergy, synergized in a socialist register to deliver the fundamental resources of health and well-being to the economically dispossessed (Heller, 2000). The aftermath of 2008 financial crisis saw a reduction of investment in public health in many countries including across India; however the investment in state public health infrastructure has been a consistent policy tool of the successive communist governments in Kerala. The "Kerala model of health" is often seen as "good health based on social justice and equity," rooted in the CPI(M)'s conceptualization of health as a universal human right (Ekbal, 2017).

With adequate capacity built into the state-led health care system, COVID-19 treatment in Kerala focused on delivering care in government hospitals rather than assigning it to private players. For every coronavirus case, a comprehensive route map was created. This was then widely circulated in neighborhoods and social media to find whether other people have been exposed to the virus (Rakesh, 2020). When the virus spread to hundreds, healthcare workers and volunteers with flowcharts conducted rigid contact tracing and thus helped to flatten the curve of community spreading. The community participation was evident when students also chipped in forming walk-in kiosks for taking samples (Varma, 2020). The counter-hegemonic use of spaces and decentralization of power was reflected when village councils enabled community outreach programmes to combat COVID-19 (Sweeney, 2020). This strong community participation was equipped with a strong public education system that had invested in literacy, and especially science literacy. The CPI(M)-associated civil society-led science communication program has invested into building an open science infrastructure across communities in Kerala, anchored in the concept of creating democratic spaces for universal access to scientific literacy.

Regular press meets by the Kerala government kept people well-informed about the situation. The campaign, "Break the Chain" was initiated by the Kerala government to keep people informed about the benefits of washing hands to stop the spread of the virus and thus breaking the chain of community spreading (Bhattacharya, 2020; Flattening the curve: Lessons from Kerala, 2020). A veritable group of volunteers, local government institutions, Kudumbashree, another community initiative, engaged in the production of masks and sanitizers and distribution of foods. Activities ranged from production of over 1,45,000 masks and 2,000 l of hand sanitizers, distribution of food, medicine and so on (Mohan, 2020). These are exemplary examples of workers power, to tackle the crisis of neo-liberalism.

Community Care

The mortalities resulting from COVID-19 has taken a back seat as the state of Kerala was hell-bent in sheltering its population from the catastrophe. On March 19, 2020, a 2.6 billion package was announced by the Kerala government to tackle the pandemic (Agarwal, 2020). Community kitchens, reflecting community care and ownership, were set up to feed the needy. Free treatments were provided to the infected and quarantined people. Regardless of category free rice and other essential items were distributed through ration shops. The government ensured that midday meals will be delivered for school children at their homes and social security pensions for 2 months were given to the elderly (Nowrojee, 2020). Helplines were developed for people struggling to cope with COVID-19 stress and anxiety. The state

also was equipped to bring its international migrants who were stranded in different pockets of the world and to provide them medical assistance as needed (Mathew, 2020).

COVID-19 deprived the migrant laborers of their livelihood, who came from rural areas to find work in cities. The neoliberal forces extracted the most from these migrant laborers in the form of cheap labor and during the COVID-19 pandemic they were left on the streets, exposed and vulnerable. Across Indian cities, several incidents were reported where precarious migrant workers were discriminated, marginalized, mistreated and attacked (Dutta, 2020d). The plight of migrant workers in other states were deplorable as they underwent starvation, police violence and they had to walk hundreds of kilometers to their villages from the big cities. Kerala has outshined the rest of India, in handling the plight of migrant workers. Whereas, Kerala has acknowledged that the foundation of Kerala's infrastructure constitutes the labor of precarious migrant workers from other states. The migrant workers in Kerala, from other states, were given the title of "guest workers," given proper shelters and provided with food (Why Kerala is a home to 'outsiders' - Times of India., 2020). In Kerala, several programmes were launched to help low-wage migrant workers in contrast to other states. The communist led government intervened to lessen the hardships of migrant workers in Kerala, by cash transfer, supplying free rations and food. Out of the 23,567 camps set up for the migrant workers in India, Kerala attributed for 65% (Sadanandan, 2020). The government recognized that the sudden lockdown must have caught the migrant laborers off guard and hence, took the responsibility of them in the time of uncertainty. When the stories of neglected marginalized migrant workers in countries like Singapore are surfacing (Ratcliffe, 2020), the Kerala model, shows how health is intertwined in participation, transparency and voice.

Community-centric approach by Kerala demonstrates that it has disruptive potential to break from the chains of a system embedded in individualism and create a response based on solidarity and compassion. In a world, where humans are devalued, and happiness is measure by wealth and economic growth; such models are pertinent to overcome the neoliberal dogmas. We all need a voice so that we can denounce the callous neglect for human life in the neoliberal regimes. As the so called "global leaders" like the USA, the UK, stands still, there is a clarion call to relook policymaking and regulatory processes and to adopt a people-centric method, which is the most prudent alternative we have amid the COVID-19 crisis.

Maori Organizing in Aotearoa, Power, and Politics

This paper explores Iwi-led checkpoints as a humanitarian, cultural and community response to COVID-19 against the backdrop of the colonial settler state and amidst the politics, police and power structures in Aotearoa, New Zealand. Given the historical accounts of past pandemics in Aotearoa, Māori, particularly in geographically isolated areas, have lived memories of harrowing pandemics that have been passed down through the generations. Māori collectivization to prevent virulent death once again sweeping through their communities was a necessary response. Ngata (2020) contextualizes the Iwi-led checkpoints on the borders of tribal boundaries amidst public criticism, backed by a petition against the checkpoints, created by a far right racist group called Hobson's Pledge (Dalder, 2019). Ngata (2020) explains the importance of the checkpoints to safeguard isolated communities because "nobody will come to do this for us, and nor could they do it as effectively as us, for nobody knows and loves our people and place as we do (para 8)."

Iwi-Led Checkpoints

Te Whānau a Apanui, located in a geographically isolated area in the Eastern Bay of Plenty, took prompt action to close their borders with *Karakia* (Prayer) to all who did not reside in the area on 25 March 2020. Waititi (2020), *Iwi* (Tribal) representative announced the establishment of an Iwi checkpoint citing around 200 Kaumātua (Elders) residing in their Tribal area, sparce medical services and the over 100 km journey to the nearest hospital as compelling factors to exert their sovereignty to look after their own community. There were no COVID-19 cases in the area and the Iwi was committed to keeping the virus locked out. To reduce the travel to supermarket services, also located over 100 km away, Te Whānau a Apanui started an online shopping system providing the community with supermarket food 2 days a week (Paranihi, 2020). Whilst their major food sources are derived from the forest, the ocean and rivers, the government uniformly banned all hunting and fishing. Notwithstanding the ban, Waititi encouraged other Māori communities not to "rely on the government and their supplies to save us" (Waititi, 2020 as cited in Paranihi, 2020, para 11) and continue with their tribal food gathering practices.

The remote community of Te Araroa is a 3 h drive to the nearest hospital in Gisborne. The Iwi also created plans to establish their checkpoint prior to lockdown. Iwi in the Far North, Taranaki, Maketu, and Murupura followed suit (Graham-McClay, 2020; Jones, 2020; New Zealand Herald, 2020; Wright, 2020a). It was the Iwi-led checkpoint in Murupara that attracted the most attention. Two gang members from different gangs joined the frontline Iwi-led checkpoint drawing negative and racist criticism (Judd, 2020 as cited in Hurihanganui, 2020; Ngarewa-Packer, 2020). In response to the racism and cited in *Video: Gangs unite with Iwi against COVID-19* (Peters K. N., 2020), Iwi checkpoint organizer, Leila Rewi created a Tiktok video of the checkpoint volunteers—Iwi, Māori from other areas and gang members, united and dancing on checkpoint duty to a song called *Tutahi - Stay* (Coddington et al., 2020), by a collection of New Zealand artists, innovatively recorded during lockdown level four. The Tiktok video went viral, shared by supporters and critics. Noteably the negative volume on Iwi-led checkpoints increased as Hobson's Pledge initiated a petition to stop the checkpoints. Conversely an outpouring of support for the checkpoints not just by Iwi members but by general community members gathered momentum (One Double, 2020; Scoop, 2020). The Iwi-led checkpoint at Murupura continued unabated, with frontliners smiling and, or dancing as they exercised their Iwi and community sovereignty. Mongrel Mob member, Deets Edwards explains that:

> we weren't breaking the law, we were out there greeting people with hello. We didn't physically stand there to force someone to stop. They stopped on their own free will…The people that didn't know me, like everyone else, they'll judge a book by its cover (Edwards, 2020 as cited in Wright, 2020b, paras 7–8).

The Iwi-led checkpoints continued to be emphatically discussed in the Epidemic Response Committee's live online daily meetings, led by the leader of the National Party, Simon Bridges. This committee was set up by the government to scrutinize its national COVID-19 pandemic response. Recounting reports from concerned citizens annoyed that their freedom of movement was being challenged at Iwi-led checkpoints, the committee demanded answers from the Police Commissioner, Andrew Coster about the legality of these checkpoints. Under lockdown level four regulations, everyone was banned from traveling inter-regionally (Small, 2020); but that didn't stop some people, who attempted travel under the cover of darkness (Canning, 2020; Neilson, 2020).

During the first few weeks of lockdown level four, at the local level, Police supported the checkpoints both conceptually and visibly (Peters K. N., 2020; Peters M., 2020; RNZ, 2020). This was backed by the Deputy Police Commissioner, Wally Haumaha who was keen to model community partnerships with Iwi, particularly in isolated areas (Haumaha, cited in Peters K. N., 2020). Initially, when the Media asked the PM about whether she approved of the checkpoints, described by the Media as "medical checkpoints," the PM sidestepped the issue and responded generally about self-isolation as evidenced in both the live-update video recordings and the transcriptions of those video recordings uploaded on the official New Zealand government website (New Zealand Government, 2020a). The next time the Iwi-led checkpoints were raised by Media during the PM's live updates was on day four of lockdown on 29 March 2020. Again, the PM's response carefully sidestepped referencing "Iwi-led checkpoints," referring instead to her communication with local MPs from the Iwi area regarding the local establishment of roadblocks (New Zealand Government, 2020b). As criticism mounted in some sectors regarding the legality of the Iwi-led checkpoints, the PM was further questioned about her stance on the checkpoints almost 1 month later on 22 April 2020. The PM indicated that the Police have been working with communities to ensure that the checkpoints are conducted within the law, strategically keeping the focus on the intent and response of communities to safeguard each other. An acknowledgment from the PM that the checkpoint initiatives from these communities were Iwi-led was still not forthcoming (New Zealand Government, 2020c). The next day on 23 April 2020, the verbatim was not that different, except the PM highlighted that the powers to stop people lay only with the Police and the Civil Defense (New Zealand Government, 2020d).

New Police Commissioner, Coster (2020) followed suit by carefully avoiding the phrase "Iwi-led checkpoints," in his article, referring to them instead as "COVID-19 community checkpoints," or "community-led checkpoints" devaluing the stoic, around the clock labor actioned by Iwi in placing their bodies on the line to prevent the spread of COVID-19 amongst their Whānau, Hapu, Iwi and communities. Moreover, Coster added that "a strong enforcement-led response to the [Iwi] checkpoints could lead to protests at various sites around the country…" (Coster, 2020, para 9). Iwi members gathering and protesting at various sites around the country during lockdown level four would disrupt the lockdown plan and not align with the government's messaging that "we are all in this together." Coster added that the model of policing underscored by the principle of discretion directed Police action by deploying Police to Iwi-led checkpoints in a monitoring capacity to both ensure that public movement is lawful and within the lockdown restrictions. Notably, the discretion principle that underpins the policing model referred to by Coster was not utilized during the latter half of 2019 when the Police presented in considerable numbers at Ihumātao to restrict the movement of the land protectors occupying the land (Webb-Liddall, 2019). The communication strategy adopted by the PM and the Police Commissioner rendered taboo the mere mention of "Iwi-led checkpoints" in their media statements, preferring instead the referencing of "community checkpoints." Whilst there were reports of community volunteers assisting at the Iwi-led checkpoints, these care initiatives were, as the name clearly describes, led by Iwi. Yet an examination of the transcriptions reveal that the government had no qualms about referencing Iwi when Iwi were assisting Civil Defense teams to distribute food among vulnerable communities and collaborate with agencies to secure housing assistance (New Zealand Government, 2020d). When the pulse of racism quickens to sideline Iwi sovereignty, exacerbated by negative public opinion, the government's mantra that "we are all in this together" during the COVID-19 pandemic reveals the entrenched logics of acceptable participation determined by this nation's hegemonic, colonial power structures.

Ironically, while some of New Zealand's concerned citizens together with National and Act Party members on the Epidemic Response Committee were challenging the legality of the Iwi-led checkpoints, a judicial review application challenging the legality of some of the lockdown orders was before the High Court. In Christiansen v. The Director-General of Health (2020), the applicant, Oliver Christiansen returned to New Zealand to visit his dying father in April 2020. Christiansen was confined to managed isolation but when his father's illness suddenly declined, Christiansen sought an exemption to the order issued under the Health Act 1956, as a matter of urgency, to enable him to visit his father. Ministry of Health officials acting under delegated authority by the Director-General, Dr. Ashley Bloomfield declined Christiansen's application. The High Court upheld Christiansen's application and overruled the government's COVID-19 lockdown orders (Hurley and Bayer, 2020). The High Court ruled that according to the Order provisions, the Ministry of Health was wrong in denying Christiansen an early exemption out of mandatory lockdown to visit his father. Christiansen was able to visit his father 1 day before he passed away. Subsequently Jacinda Ardern, prime minister ordered a review into all the Ministry of Health decisions on this matter.

Former Parliamentary Counsel, Andrew Borrowdale is also seeking a judicial review by the High court to determine whether

the government was sufficiently empowered by legislation to enact lockdown levels four and three. Chair of the Epidemic Response Committee, Simon Bridges has tagged onto the legal debacle and is planning to tackle parliamentary privilege to summon government officials and parliamentary privilege to obtain all legal advice received by the government to enact lockdown to determine if the government acted within its powers (Geddis, 2020; Geiringer, 2020; RNZ, 2020). Obtained via public official information requests, thousands of official COVID-19 government papers were released on 8 May 2020 revealing the basis for the government's COVID-19 decisions (Walls and Cheng, 2020), except for the legal advice documents.

DISCUSSION

One of the key threads that emerges from the community organizing in response to COVID-19 is the nature of organizing work as political. Interrogating and disrupting the depoliticization of communities to be incorporated into community-based participatory projects serving the agendas of capital, community organizing at the margins in the Global South/South in the North foreground the concept of community sovereignty. Community sovereignty, moves beyond the concept of community mutual aid in support of each other, to organizing communities to resist local-global structures of individualization and privatization under the hegemonic neoliberal project (Dutta, 2016). Organizing processes and structures of pandemic response, owned by those at the "margins of the margins" in communities challenge the hegemonic theorizing of pandemic response that construct the prevention of the pandemic in terms of behavior change (constructing individuals in the dichotomous category of adherence/non-adherence).

In resistance to a top-down definitional framework that sees behavior change as individual response produced through persuasive messages (Dutta, 2005), community sovereignty foregrounds and renders visible the structures of inequality that are exacerbated and worked upon by the pandemic, constituting the contexts within which behaviors are enacted. Health behavior, in this case COVID-19-related behaviors, Therefore, in the two cases offered in this article, when the very structural formations that constitute behaviors are targeted, health communication works toward structural transformation that enables collective preventive behaviors at the community level. In addition, community organizing is directed specifically at addressing the overarching structures, thus directly addressing the health needs at the margins and seeking to transform overarching pandemic inequalities. Based on these two cases, we attend to the interplays of community and state structures in constituting pandemic response, mediated through voice (see **Figure 1**). Voices of the margins laying claims to infrastructures of health, education, income, and food interact with the state, both constituted by the state and in turn, constitutive of it. Drawing on the key conceptual tenets of the CCA, we thus propose a strong state for pandemic response that is simultaneously centralized and de-centralized. As opposed to the extreme neoliberal state that is "rolled out" to serve capital while simultaneously being "rolled back" from the delivery of essential health and well-being infrastructures, the culture-centered state is strengthened in its capacity to deliver substantive health and well-being infrastructures through the participation of the "margins of the margins."

In India, the community response in Kerala is situated within a socialist structure of organizing politics and economics, with the strong presence of worker organizing and the transformative role of the CPI(M). The specific forms of welfare and workplace protections secured in the state through the ongoing organizing of unions and collective movements serve as the basis of developing a COVID-19 response that is directed at addressing the entrenched structural inequalities. The recognition of structural violence as the conduit through which the pandemic spreads, the community organizing work complements the structurally-directed intervention designed by the CPI(M). Drawing on the deep roots of grassroots organizing, land reforms and resource distribution that form the architectures of the CPI(M), community organizing works alongside Left party politics to materialize a socialist framework of pandemic response. The community response in Kerala is constituted by the state policy directed at decentralization and community-led governance. Contrast the Kerala model with the pandemic response across India formulated within a neoliberal framework, without protection for the poor and the working classes, expelling migrant workers into conditions of vulnerability, and without the provision of fundamental resources of health and well-being (Roy, 2020). Moreover, contrast the Kerala model of a strong education infrastructure with robust science literacy in the backdrop of a weak education and science infrastructure across large cross-sections of neoliberal idea, with the ruling Hindutva forces being key players in the dissemination of misinformation. The strong scientific temperament in community networks in Kerala stands in contrast to the communicative structures of disinformation and superstition disseminated by Hindutva forces across India.

In Aotearoa, iwi-led checkpoints, grounded in the voices and actions of grassroots Whānau, Hapu, and Iwi, foreground the concept of community sovereignty (tino rangatiratanga) in the backdrop of a settler colonial state. Through sovereignty, communities take collective ownership of households, families, and larger collectives, anticipating and addressing the deep inequalities that are likely to impact disproportionately communities at the margins. The larger racist responses to iwi-led checkpoints, particularly from the right (National Party, center right), using the language of law and order, depict the ways in which transformative community participation challenges the hegemonic formations of colonial-capitalist state structures. Through their participation in organizing responses to the pandemic that assert materialities of boundary-making to protect community health and well-being, iwi-led checkpoints write new possibilities for organizing global health and well-being. The organizing of community voice is constituted

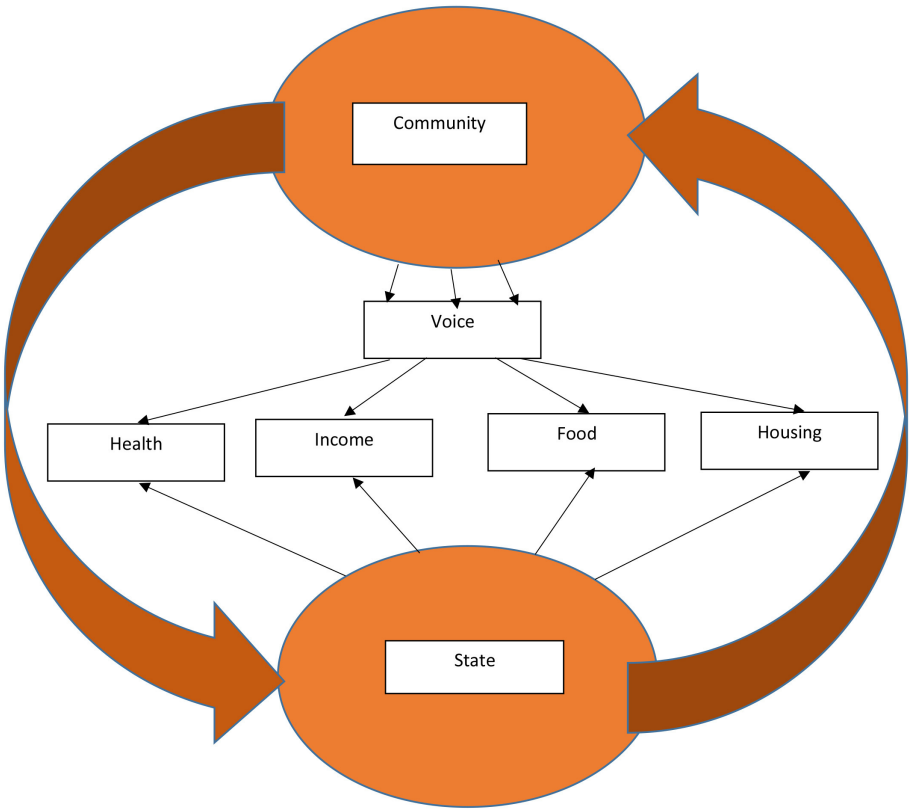


FIGURE 1 | Community-state interaction in constituting COVID-19 response.

amidst the Te Tiriti O Waitangi (Treaty of Waitangi) that offers a framework for making Māori claims into the colonial state. The Treaty itself emerges in the discursive-material arena as a register for challenging the catalytic expansion of neoliberalism. Moreover, the check-point response is constituted amidst a center-Left Labor-led government that has offered a strong centralized response to the pandemic, driven by science and addressing the economic needs of those hit by the lock-down. Particularly salient in Labour's response to the pandemic are clear communication and the overarching narrative of kindness that shaped the response to the pandemic. Māori community sovereignty is negotiated in dialogue with a state that is currently turning toward a rights-based response in contrast to the earlier capital-friendly leadership of the National Party (Note here that the neoliberal reforms were initiated into New Zealand by Labor and pursued aggressively subsequently by National Party as well as Labor). Foregrounding the logics of community sovereignty in global responses to COVID-19 recognizes community agency as the basis of pandemic response. Community participation in this instance, rather than being directed and scripted by the colonial state to fit pre-existing colonial agendas, emerges as a site of owning community health and well-being. Voices reflecting Māori agency exist in ongoing negotiations of power with the colonial state, foregrounding the vitality of Māori party formations in shaping state responses. Simultaneously, given the large scale health disparities experienced by Māori in New Zealand, these party formations ought to be fundamentally anchored in commitments to securing universal rights to basic income, housing, food, education, and health.

In summary, the culture-centered processes of community organizing drawn on the case studies of community organizing in Communist Kerala and in iwi-led Māori checkpoints in settler colonial Aotearoa New Zealand foreground the vital work of alternative[4] practices of health response, serving as the basis for robust alternative imaginations amid the pandemic. Both of these contextually situated frameworks of pandemic response recognize and work with a radical conceptualizing of community organizing, owned by the "margins of the margins" as the basis of transforming the deep inequalities that threaten human health and well-being, and that have been rendered visible by the pandemic. Compared to the failed neoliberal responses elsewhere across the globe, we point to the exceptional success of the Kerala model and the Māori model. The exceptionalism of these models offers an imaginary anti-dote to exceptional neoliberalism that

[4]The very notion of "alternative" is constituted in relationship to the neoliberal model. In this article, we hope that these alternatives emerge as pathways to the mainstream in post-COVID organizing.

has worked over the last three decades by turning the structural and material violence of neoliberalism into the normative mode of global governance. The interplays of socialist organizing and community democracy materially evident in the cases discussed here dismantle the neoliberal ideology that constitutes the exceptional violence evident globally amidst the pandemic, both as a result of the pandemic as well as a result of the market-driven policy responses to pandemic. The very processes of community participation that co-opt community agency into serving the neoliberal status quo are organized in resistance in these two instances, thus challenging the various forms of erasure written into hegemonic processes of knowledge production and health response. Cultural centering is the turn toward communities as spaces for challenging structures and building communicative equality through grassroots democracy. Co-creating infrastructures for community voices centers grassroots democracy while simultaneously re-organizing the state in socialist principles to ensure fundamental access to education, income, housing, food, health and well-being. Although the two examples offered here provide openings for building democratic socialist pandemic responses, more broadly, they offer global registers for re-organizing health, education, housing, food, and income. Drawing on Roy's (2020) invitation to work through the "pandemic as a portal," our analysis of community organizing in Kerala and community-led Māori checkpoints in Aotearoa New Zealand puts forth a conceptual register for addressing COVID-19 transitions, as well as for organizing post-COVID political economies. Ultimately, communicative equality as the basis for health communication is constituted in ongoing dialogue between community agency and state response, seeking to building infrastructures for voices of the "margins of the margins" and simultaneously creating a socialist state.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

All authors have conducted literature review, participated in theorizing, and written part of the manuscript.

FUNDING

This work was funded by the Center for Culture-Centered Approach to Research and Evaluation (CARE).

REFERENCES

Agarwal, S. (2020, May 2). This state in India shows us why fighting COVID-19 requires working-class power. *Truthout*. Available online at: https://truthout.org/articles/this-state-in-india-shows-us-why-fighting-covid-19-requires-working-class-power/

Ahmed, F., Ahmed, N., Pissarides, C., and Stiglitz, J. (2020). Why inequality could spread COVID-19. *Lancet Public Health* 5:e240. doi: 10.1016/S2468-2667(20)30085-2

Basu, A., and Dutta, M. J. (2009). Sex workers and HIV/AIDS: analyzing participatory culture-centered health communication strategies. *Hum. Commun. Res.* 35, 86–114. doi: 10.1111/j.1468-2958.2008.01339.x

Bhatt, S. C., and Bhargava, G. K. (2005). *Land and People of Indian States and Union Territories: (in 36 volumes)*. New Delhi: Gyan Publishing House.

Bhattacharya, S. (2020, March 16). Kerala launches break the chain handwashing campaign in light of coronavirus pandemic. *Republic World*. Available online at: https://www.republicworld.com/india-news/general-news/kerala-launches-break-the-chain-handwashing-campaign-against-covid-19.html

Biswas, S. (2020, April 16). How India's Kerala state 'flattened the curve'. *BBC News*. Available online at: https://www.bbc.com/news/world-asia-india-52283748

Brenner, N., Peck, J., and Theodore, N. (2010). Variegated neoliberalization: geographies, modalities, pathways. *Glob. Netw.* 10, 182–222. doi: 10.1111/j.1471-0374.2009.00277.x

Cammaerts, B. (2020). The neo-fascist discourse and its normalisation through mediation. *J. Multicult. Discourses* 15, 1–16. doi: 10.1080/17447143.2020.1743296

Canning, R. (2020, April 7). Turangi population swells, bach owners arrive in dead of night. *NZ Herald*. Available online at: https://www.nzherald.co.nz/rdp-taupo-turangi/news/article.cfm?c_id=1503734&objectid=12323243

Chapple, S. (2016). New Zealand's first recorded post-contact epidemic: untangling the tale of Rongotute and Te Upoko o Rewarewa. *Archaeol. N. Zeal.* 21–32.

Chapple, S. (2018). *Death and Disease at the Dawn of New Zealand's History*. ResearchGate. Available online at: https://www.researchgate.net/profile/Simon_Chapple5/publication/327427166_Death_and_disease_at_the_dawn_of_New_Zealand's_history/links/5b8ee7e7299bf114b7f60bfd/Death-and-disease-at-the-dawn-of-New-Zealands-history.pdf

Chasin, B. H., and Franke, R. W. (1991). Kerala state, India: Radical reform as development. *Month. Rev.* 42:1. doi: 10.14452/MR-042-08-1991-01_1

Checkpoint (2020, March 26). Covid-19: Does NZ have enough ICU beds, ventilators? *RNZ*. Available online at: https://www.rnz.co.nz/national/programmes/checkpoint/audio/2018740328/covid-19-does-nz-have-enough-icu-beds-ventilators

Chetterje, P. (2020). Gaps in India's preparedness for COVID-19 control. *Lancet Infect. Dis.* 20:544. doi: 10.1016/S1473-3099(20)30300-5

Christiansen v. The Director-General of Health (2020). NZHC. 887. Available online at: https://courtsofnz.govt.nz/assets/cases/Christiansen-v-The-Director-General-of-Health-Reasons-NZHC-887.pdf

Coddington, A., Wiley, B., Smith, H., Hall, R., Theia., Kora, F., et al. (2020, April 14). *Tutahi – Stay [Official Music Video]*. Available online at: https://www.youtube.com/watch?v=rVhd21jpjGQ

Coster, A. (2020, May 4). Coronavirus: Iwi checkpoints were about safety and discretion. *Stuff*. Available online at: https://www.stuff.co.nz/national/health/coronavirus/121400826/coronavirus-iwi-checkpoints-were-about-safety-and-discretion

Dalder, M. (2019, February, 2). The furious world of New Zealand's far right nationalists. *Spinoff*. Available online at: https://thespinoff.co.nz/politics/31-12-2019/summer-reissue-the-furious-world-of-new-zealands-far-right-nationalists/

Day, A. (1999). 'Chastising its people with scorpions:' Māori and the 1913 smallpox epidemic. *N. Zeal. J. Hist.* 33, 180–199.

Dreze, J., and Sen, A. (2002). India: Development and Participation. Oxford University Press on Demand.

Dutta, M. J. (2004). The unheard voices of Santalis: communicating about health from the margins of India. *Commun. Theor.* 14, 237–263. doi: 10.1111/j.1468-2885.2004.tb00313.x

Dutta, M. J. (2005). Theory and practice in health communication campaigns: a critical interrogation. *Health Commun.* 18, 103–122. doi: 10.1207/s15327027hc1802_1

Dutta, M. J. (2007). Communicating about culture and health: theorizing culture-centered and cultural sensitivity approaches. *Commun. Theory* 17, 304–328. doi: 10.1111/j.1468-2885.2007.00297.x

Dutta, M. J. (2008). *Communicating Health: A Culture-Centered Approach*. Cambridge: Polity Press.

Dutta, M. J. (2011). *Communicating Social Change. Structure, Culture and Agency*. New York, NY: Routledge.

Dutta, M. J. (2013). Public relations in a global world: culturally centering theory and praxis. *Asia Pac. Public Relat. J.* 14, 21–31.

Dutta, M. J. (2015). Decolonizing communication for social change: a culture-centered approach. *Commun. Theor.* 25, 123–143. doi: 10.1111/comt.12067

Dutta, M. J. (2016). *Neoliberal Health Organizing: Communication, Meaning, and Politics*. New York, NY: Routledge.

Dutta, M. J. (2017). Migration and health in the construction industry: culturally centering voices of Bangladeshi workers in Singapore. *Int. J. Environ. Res. Public Health* 14:132. doi: 10.3390/ijerph14020132

Dutta, M. J. (2018). Culture-centered approach in addressing health disparities: communication infrastructures for subaltern voices. *Commun. Methods Meas.* 12, 239–259. doi: 10.1080/19312458.2018.1453057

Dutta, M. J. (2019). What is alternative modernity? decolonizing culture as hybridity in the Asian Turn. *Asia Pac. Med. Educ.* 29, 178–194. doi: 10.1177/1326365X19881256

Dutta, M. J. (2020a). "Culture-centered community-led testing," in *CARE White Paper* (Palmerston North: Center for Culture-Centered Approach to Research and Evaluation).

Dutta, M. J. (2020b). "A culture-centered approach to pandemic response: voice, universal infrastructure, and equality," in *CARE White Paper* (Palmerston North: Center for Culture-Centered Approach to Research and Evaluation).

Dutta, M. J. (2020d). COVID-19: India's underclasses and the depravity of our unequal societies. *The Citizen*. Available online at: https://www.thecitizen.in/index.php/en/NewsDetail/index/4/18516/COVID19----Indias-Underclasses-and-the-Depravity-of-Our-Unequal-Societies

Dutta, M. J. (2020e). "Infrastructures of housing and food for low-wage migrant workers in Singapore," in *CARE White Paper* (Palmerston North: Center for Culture-Centered Approach to Research and Evaluation).

Dutta, M. J., and Basu, A. (2008). Meanings of Health: Interrogating structure and culture. *Health Commun.* 23, 560–572. doi: 10.1080/10410230802465266

Dutta, M. J., and de Souza, R. (2008). The past, present, and future of health development campaigns: Reflexivity and the critical-cultural approach. *Health Commun.* 23, 326–339. doi: 10.1080/10410230802229704

Dutta, M. J., Pandi, A. R., Mahtani, R., Falnikar, A., Thaker, J., Pitaloka, D., et al. (2019). Critical health communication method as embodied practice of resistance: culturally centering structural transformation through struggle for voice. *Front. Commun.* 4:67. doi: 10.3389/fcomm.2019.00067

Dutta,M. J. (2020c). "Structural constraints, voice infrastructures, and mental health among low-wage migrant workers in Singapore: solutions for addressing COVID-19," in *CARE White Paper* (Palmerston North: Center for Culture-Centered Approach to Research and Evaluation).

Ekbal, D. B. (2017). Kerala Model of Health: From Success to Crisis. The New Indian Express. Available online at: https://www.newindianexpress.com/states/kerala/2017/aug/14/kerala-model-of-health-from-success-to-crisis-1642904.html

Flattening the curve: Lessons from Kerala. (2020, May 6). *Punch Newspapers*. Available online at: https://punchng.com/flattening-the-curve-lessons-from-kerala/

Franke, R. W., and Chasin, B. H. (1992). Kerala state, India: radical reform as development. *Int. J. Health Serv.* 22, 139–156. doi: 10.2190/HMXD-PNQF-2X2L-C8TR

Geddis, A. (2020, March 25). How politics, police and power work in lockdown New Zealand. *The Spinoff*. Available online at: https://thespinoff.co.nz/politics/25-03-2020/how-politics-police-and-power-work-in-lockdown-new-zealand/

Geiringer, C. (2020, May 6). Was covid-19 lockdown legal? Professor Claudia Geiringer explains. *RNZ*. Available online at: https://www.rnz.co.nz/national/programmes/checkpoint/audio/2018745408/was-covid-19-lockdown-legal-professor-claudia-geiringer-explains

Graham-McClay, C. (2020, March 23). New Zealand's Māori tribes set up checkpoints to avoid 'castrophic' deaths. *The Guardian*. Available online at: https://www.theguardian.com/world/2020/mar/24/new-zealands-Maori-tribes-set-up-checkpoints-to-avoid-catastrophic-coronavirus-deaths/

Gustafsson, J. E. (2018). Bentham's binary form of maximising utilitarianism. *Br. J. History Philos.* 26, 87–109. doi: 10.1080/09608788.2017.1347558

Habersaat, K. B., Betsch, C., Danchin, M., et al. (2020). Ten considerations for effectively managing the COVID-19 transition. *Nat. Hum. Behav.* 4, 677–687. doi: 10.1038/s41562-020-0906-x

Heller, P. (2000). Degrees of democracy: some comparative lessons from India. *World Polit.* 52, 484–519. doi: 10.1017/s0043887100020086

Heller, P., Harilal, K. N., and Chaudhuri, S. (2007). Building local democracy: evaluating the impact of decentralization in Kerala, India. *World Dev.* 35, 626–648. doi: 10.1016/j.worlddev.2006.07.001

Heller, P., and Isaac, T.T. (2005). "The politics and institutional design of participatory democracy: lessons from Kerala, India," in *Democratizing Democracy: Beyond the Liberal Democratic Canon*, eds B. D. Santos (London: Verso), 405–446.

Hickey, B. (2020, March 14). We must go hard and we must go early. *Newsroom*. Available online at: https://www.newsroom.co.nz/2020/03/14/1083045/we-must-go-hard-and-we-must-go-early

Hurihanganui, T. A. (2020, May 1). MPs' questioning of legal iwi checkpoints 'really is racism.' *RNZ*. Available online at: https://www.rnz.co.nz/news/te-manu-korihi/415617/mps-questioning-of-legal-iwi-checkpoints-really-is-racism

Hurley, S., and Bayer, K. (2020, May 5). Covid 19 coronavirus: Judge overrules lockdown and allows son to visit dying Dad, PM orders review of refusals. *NZ Herald*. Available online at: https://www.nzherald.co.nz/nz/news/article.cfm?c_id=1&objectid=12329451

Jones, C. (2020, April 7). Ngāti Manawa set up checkpoints due to lockdown rule breakers. *RNZ*. Available online at: https://www.rnz.co.nz/news/te-manu-korihi/413695/ngati-manawa-set-up-checkpoints-due-to-lockdown-rule-breakers

Kurian, O. C. (2020, April 24). How the Indian state of Kerala flattened the coronavirus curve | Oommen C Kurian. *The Guardian*. Available online at: https://www.theguardian.com/commentisfree/2020/apr/21/kerala-indian-state-flattened-coronavirus-curve

Malliori, M., Golna, C., Souliotis, K., and Hatzakis, A. (2013). Financial crisis, austerity, and health in Europe. *Lancet* 382:392. doi: 10.1016/S0140-6736(13)61664-5

Mannathukkaren, N. (2013). The rise of the national-popular and its limits: Communism and the cultural in Kerala. *Inter Asia Cult. Stud.* 14, 494–518. doi: 10.1080/14649373.2013.831160

Masih, N. (2020, April 10). *The Washington Post*. Available online at: https://www.washingtonpost.com/world/aggressive-testing-contact-tracing-cooked-meals-how-the-indian-state-of-kerala-flattened-its-coronavirus-curve/2020/04/10/3352e470-783e-11ea-a311-adb1344719a9_story.html

Mathew, S. P. (2020, May 9). COVID-19 in Kerala: staying ahead of the curve. *NewsClick*. Available online at: https://www.newsclick.in/COVID-19-Kerala-Highest-Recovery-Rate-Pandemic

Ministry of Health (2020, March 23). *36 New Cases of COVID-19 in New Zealand*. Ministry of Health. Available online at: https://www.health.govt.nz/news-media/media-releases/36-new-cases-covid-19-new-zealand

Mohan, S. (2020, April). Kudumbashree sews success in times of COVID-19. *The New Indian Express*. Available online at: https://www.newindianexpress.com/good-news/2020/apr/07/kudumbashree-sews-success-in-times-of-covid-19-2126644.html

Mounk, Y. (2020, March 11). The extraordinary decisions facing Italian doctors. *The Atlantic*. Available online at: https://www.theatlantic.com/ideas/archive/2020/03/who-gets-hospital-bed/607807/

Nag, M. (1983). Impact of social and economic development on mortality: comparative study of Kerala and West Bengal. *Econ. Polit. Wkly.* 18, 877–900.

Nair, K. R. (2007). "Governance reforms and development in Kerala in the context of globalization," in *Globalisation, Governance Reforms and Development in India* (New Delhi: SAGE Publications).

Namboodiripad, E. M. S. (1984). *Kerala, Society and Politics: an Historical Survey*. National Book Centre.

Namboodiripad, E. M. S. (1994). *The Communist Party in Kerala: Six Decades of Struggle and Advance*. National Book Centre.

Neilson, N. (2020, April 9). COVID-19 coronavirus: 'Stay away' plea as small towns brace for Easter influx. *NZ Herald*. Available online at: https://www.nzherald.co.nz/nz/news/article.cfm?c_id=1&objectid=12323983

New Zealand Government (2020a). *Prime Minister's Press Conference*. [250320 PM daily covid-19 press conference.pdf]. Beehive.govt.nz. Available online at: https://www.beehive.govt.nz/feature/covid-19-updates (accessed March 25, 2020)

New Zealand Government (2020b). *Post-cabinet Press Conference*. [290320 PM and all of govt joint press conference.pdf]. Beehive.govt.nz. Available online at: https://www.beehive.govt.nz/feature/covid-19-updates (accessed March 29, 2020)

New Zealand Government (2020c). *Prime Minister and Director-General of Health Press Conference*. [2204 PM and DG of health daily covid-19 press conference.pdf]. Beehive.govt.nz. Available online at: https://www.beehive.govt.nz/feature/covid-19-updates (accessed April 22, 2020)

New Zealand Government (2020d). *PM and All of Government Press Conference*. [2304 All of government press conference.pdf]. Beehive.govt.nz Available online at: https://www.beehive.govt.nz/feature/covid-19-updates (accessed April 23, 2020)

New Zealand Herald (2020, April 27). Taranaki iwi community checkpoint plans start tomorrow. *NewstalkZB*. Available online at: https://www.newstalkzb.co.nz/news/national/taranaki-iwi-community-checkpoint-plans-start-tomorrow/

Ngarewa-Packer, D. (2020, May 1). *LIVE With Our Co-Leader and Te Tai Hauāuru Candidate Debbie Ngarewa-Packer, and Former New Plymouth Mayor and Self-Described 'Recovering Racist' Andrew Judd. We're Talking About Racism, Pākehā Fragility and Māori Leadership in the Age of COVID-19*. #MāoriConversations [Video]. Facebook. Available online at: https://www.facebook.com/debbiengarewapacker/videos/2349205044\penalty-\@M86539

Ngata, T. (2020, March 7). COVID-19 and the Māori duty to protect. *Overland*. Available online at: https://overland.org.au/2020/05/covid-19-and-the-Maori-duty-to-protect/?fbclid=IwAR14lJWn-dEhNZ5oOA5rSrCExiQq3DiMM_oq3ILLP-kMu3MMO_w6jGkg5aA/

Nowrojee, B. (2020, May 9). *How a South Indian State Flattened Its Coronavirus Curve. The Diplomat – The Diplomat Is a Current-Affairs Magazine for the Asia-Pacific, With News and Analysis on Politics, Security, Business, Technology and Life Across the Region*. Available online at: https://thediplomat.com/2020/05/how-a-south-indian-state-flattened-its-coronavirus-curve/

One Double, X. (2020, May 4). *Eastern Bay Praises Iwi-Led Cordons*. Available online at: https://www.1xx.co.nz/local-news/2020/5/4/eastern-bay-praises-iwi-led-cordons

Ovadia, D. (2020, March 13). COVID-19: What can the world learn from Italy? *Medscape*. Available online at: https://www.medscape.com/viewarticle/926777#vp_2

Paranihi, R. (2020, April 6). East coast iwi start online shopping system. *Te Ao*. Available online at: https://www.teaoMaori.news/east-coast-iwi-start-online-food-shopping-system/

Pennington, P. (2020, April 1). Covid-19: NZ sources more ventilators amid rampant global demand. *RNZ*. Available online at: https://www.rnz.co.nz/news/national/413111/covid-19-nz-sources-more-ventilators-amid-rampant-global-demand

Peters, K. N. (2020, April 19). Video: Gangs unite with Iwi against covid-19. *Newsroom*. Available online at: https://www.newsroom.co.nz/2020/04/19/1135695/video-gangs-unite-with-iwi-against-covid-19

Peters, M. (2020). Checkpoints on Coast. *The Gisborne Herald*. Available online at: http://www.gisborneherald.co.nz/local-news/20200324/protecting-the-most-vulnerable/

PTI (2020, May 9). Death toll due to COVID-19 rises to 1,981; cases climb to 59,662. *Deccan Herald*. Available online at: https://www.deccanherald.com/national/death-toll-due-to-covid-19-rises-to-1981-cases-climb-to-59662-835398.html

Raghunath, A. (2020, January 30). Coronavirus-infected Kerala girl's condition reported stable; more results pending. *Deccan Herald*. Available online at: https://www.deccanherald.com/national/coronavirus-infected-kerala-girls-condition-reported-stable-more-results-pending-799788.html

Raj, S. (2020). *An Overview of Health Care Sector in Kerala. Studies in Indian Place Names*. Available online at: https://archives.tpnsindia.org/index.php/sipn/article/view/1905

Rakesh, K. M. (2020, March 12). Retracing steps of a coronavirus-affected family. *Telegraph India | Latest News, Top Stories, Opinion, News Analysis and Comments*. Available online at: https://www.telegraphindia.com/india/retracing-steps-of-a-coronavirus-affected-family/cid/1752842

Ratcliffe, R. (2020, April 23). 'We're in a prison': Singapore's migrant workers suffer as COVID-19 surges back. *The Guardian*. Available online at: https://www.theguardian.com/world/2020/apr/23/singapore-million-migrant-workers-suffer-as-covid-19-surges-back

Reid, P., Cormack, D., and Paine, S. J. (2019). Colonial histories, racism and health: the experience of Māori and indigenous peoples. *Public Health* 172, 119–124. doi: 10.1016/j.puhe.2019.03.027

Rice, G. W. (2018). 'That terrible time:' Reflections on the 1918 influenza pandemic in New Zealand. *N. Zeal. Med. J.* 131, 6–8.

RNZ (2020, May 7). Was the government's covid-19 lockdown illegal? *Expert Explains. One News*. Available online at: https://www.tvnz.co.nz/one-news/new-zealand/governments-covid-19-lockdown-illegal-expert-explains

Roy, A. (2020). The pandemic is a portal. *Financial Times*. Available online at: https://www.ft.com/content/10d8f5e8-74eb-11ea-95fe-fcd274e920ca

Sadanandan, R. (2020). Kerala's response to COVID-19. *Indian J. Public Health* 64:99. doi: 10.4103/ijph.IJPH_459_20

Scoop (2020, May 2). *Widespread Gratitude for Roadside Checkpoints*. Available online at: https://www.scoop.co.nz/stories/AK2005/S00047/widespread-gratitude-for-roadside-checkpoints.htm

Selberg, R. (2013). Nursing in times of neoliberal change: an ethnographic study of nurses' experiences of work intensification. *Nordic J. Working Life Stud.* 3:9. doi: 10.19154/njwls.v3i2.2548

Singh, A. (2020). Status of human development index. *SSRN Electron. J.* doi: 10.2139/ssrn.3560804

Singh, P. (2011). We-Ness and welfare: A longitudinal analysis of social development in Kerala, India. *World Dev.* 39, 282–293. doi: 10.1016/j.worlddev.2009.11.025

Small, A. (2020, April 5). Covid 19 coronavirus: Bach owners relocating during lockdown ready for Easter. *NZ Herald*. Available online at: https://www.nzherald.co.nz/nz/news/article.cfm?c_id=1&objectid=12322497

Strongman, S. (2020). Covid-19 pandemic timeline: How the coronavirus started, spread and stalled life in New Zealand. *RNZ*. Available online at: https://shorthand.radionz.co.nz/coronavirus-timeline/

Stuckler, D., Reeves, A., Loopstra, R., Karanikolos, M., and McKee, M. (2017). Austerity and health: the impact in the UK and Europe. *Eur. J. Public Health* 27(suppl_4), 18–21. doi: 10.1093/eurpub/ckx167

Sweeney, S. (2020, April 23). Communist-led Kerala a model for the world in beating COVID-19. *Morning Star*. Available online at: https://morningstaronline.co.uk/article/w/communist-led-kerala-model-world-beating-covid-19

Thanickan, J. (2006). *United Kerala Movement: A Descriptive Essay*. Available online at: https://jamesthanickan.tripod.com/id18.html

Thomas, J. J., and Jayesh, M. P. (2019). "Labour market in Kerala: examining the role of industrial and employment policies," in *Globalization, Labour Market Institutions, Processes and Policies in India* (Singapore: Palgrave Macmillan), 471–498.

Tripathi, H. (2019). Higher education in Kerala: Development, equity and issues of access. *South Asia Res.* 39(3_suppl), 42S−60S. doi: 10.1177/0262728019872054

Van Lancker, W., and Parolin, Z. (2020). COVID-19, school closures, and child poverty: a social crisis in the making. *Lancet Public Health* 5, e243–e244. doi: 10.1016/S2468-2667(20)30084-0

Varma, S. (2020, April 28). Nipah lessons helped; there is no one-stroke flattening of the curve: Kerala health minister. *The Financial Express*. Available online at: https://www.financialexpress.com/lifestyle/health/nipah-lessons-helped-there-is-no-one-stroke-flattening-of-the-curve-kerala-health-minister/1941459/

Waitangi, T. (2019). *Hauora: Report on Stage One of the Health Services and Outcomes Kaupapa Inquiry (Wai 2575)*. Legislation Direct. Available

online at: https://www.waitangitribunal.govt.nz/inquiries/kaupapa-inquiries/health-services-and-outcomes-inquiry/

Waititi, R. (2020, March 20). *Te Whānau a Apanui Response to Covid-19 [Video]*. Facebook. Available online at: https://www.facebook.com/tewhanauaapanui/videos/222819735585750

Walls, J., and Cheng, D. (2020, May 8). Covid 19 coronavirus: live - government dumps thousands of pages of official covid papers. *NZ Herald*. Available online at: https://www.nzherald.co.nz/nz/news/article.cfm?c_id=1&objectid=12330612&ref=art_readmore

Webb-Liddall, A. (2019, August 6). Protectors condemn 'intimidating' increased Police presence at Ihumātao. *The Spinoff*. Available online at: https://thespinoff.co.nz/atea/06-08-2019/protestors-condemn-intimidating-increased-police-presence-at-ihumatao/

Why Kerala is a home to 'outsiders' - Times of India. (2020, April 4). *The Times of India*. Available online at: https://timesofindia.indiatimes.com/india/why-kerala-is-a-home-to-outsiders/articleshow/74977847.cms

Williams, M. (2017). Practising democratic communism: the Kerala experience. *Soc. Regist.* 54, 27–33.

Wright, K. (2020a, April 29). Māketu checkpoint offers alternatives for level 3 travellers. *Te Ao*. Available online at: https://www.teaoMaori.news/maketu-checkpoint-offers-alternatives-level-3-travellers/

Wright, K. (2020b, May 5). Murupara Mongrol Mob member responds to Hobson's Pledge petition. *Te Ao*. Available online at: https://www.teaoMaori.news/murupara-mongrel-mob-member-responds-hobsons-pledge-\penalty-\@Mpetition/

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Public Health

REVIEW
published: 29 July 2020
doi: 10.3389/fpubh.2020.00383

COVID-19: A Multidisciplinary Review

*Nour Chams[1], Sana Chams[1], Reina Badran[2], Ali Shams[3], Abdallah Araji[4], Mohamad Raad[5], Sanjay Mukhopadhyay[6], Edana Stroberg[7], Eric J. Duval[7], Lisa M. Barton[7] and Inaya Hajj Hussein[8]**

[1] Geriatric Division, Department of Internal Medicine, Beaumont Health System, Royal Oak, MI, United States, [2] Department of Internal Medicine, Wayne State University School of Medicine, Detroit, MI, United States, [3] Department of Emergency Medicine, Beaumont Health System, Royal Oak, MI, United States, [4] Department of Diagnostic Radiology, Memorial Sloan Kettering Cancer Center, New York, NY, United States, [5] Department of Cardiology, Henry Ford Health System, Detroit, MI, United States, [6] Department of Pathology, Cleveland Clinic, Cleveland, OH, United States, [7] Office of the Chief Medical Examiner, Oklahoma City, OK, United States, [8] Department of Foundational Medical Studies, Oakland University William Beaumont School of Medicine, Rochester, MI, United States

Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) is a novel coronavirus that is responsible for the 2019–2020 pandemic. In this comprehensive review, we discuss the current published literature surrounding the SARS-CoV-2 virus. We examine the fundamental concepts including the origin, virology, pathogenesis, clinical manifestations, diagnosis, laboratory, radiology, and histopathologic findings, complications, and treatment. Given that much of the information has been extrapolated from what we know about other coronaviruses including severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV), we identify and provide insight into controversies and research gaps for the current pandemic to assist with future research ideas. Finally, we discuss the global response to the coronavirus disease-2019 (COVID-19) pandemic and provide thoughts regarding lessons for future pandemics.

Keywords: SARS-CoV-2, COVID-19, coronavirus, respiratory infection, pandemic, global health

OPEN ACCESS

Edited by:
Zisis Kozlakidis, International Agency for Research On Cancer (IARC), France

Reviewed by:
Khalid Zakaria, Michigan State University, United States
Jamil Meloelain, William Carey University, United States

****Correspondence:***
Inaya Hajj Hussein
hajjhuss@oakland.edu

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Public Health

Received: *30 April 2020*
Accepted: *01 July 2020*
Published: *29 July 2020*

Citation:
Chams N, Chams S, Badran R, Shams A, Araji A, Raad M, Mukhopadhyay S, Stroberg E, Duval EJ, Barton LM and Hajj Hussein I (2020) COVID-19: A Multidisciplinary Review. Front. Public Health 8:383. doi: 10.3389/fpubh.2020.00383

INTRODUCTION

The world has witnessed numerous epidemics and pandemics that have affected thousands to millions of lives. Despite our advances in medicine and research, we continue to be challenged with new pathogens that pose a threat to human lives, global economic security, and the healthcare system. Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) is a novel coronavirus that was first identified in Wuhan, Hubei province, central China, and is responsible for the 2019-20 pandemic.

SARS-CoV-2 is the seventh coronavirus to date that is known to infect humans. This has been possible by frequent cross-species infections and occasional spillover events (1). Two of these previously identified coronaviruses were responsible for major epidemics in the past two decades; Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) also originating from China in 2002–2003 and the Middle East Respiratory Syndrome Coronavirus (MERS-CoV) originating from the Middle East in 2012 (2, 3). All three of these coronaviruses are considered zoonotic in origin and have the ability to cause severe and fatal illness in humans (3, 4). Unfortunately, given their large genetic diversity and the frequent recombination of their genomes coupled with the increase in human-animal interface activities due to modern agricultural practices, novel coronaviruses are likely to continue to develop and cause periodic seasonal spreads (3).

Here, we provide a multidisciplinary review of the current literature involving the SARS-CoV-2 virus. We review the origin of the virus, the course of disease, the therapeutic investigations, and the global response. Specifically, we discuss the pathogenesis, histopathology, virology, and immune response. We also examine the clinical manifestations, diagnosis, laboratory and radiology findings, in addition to common complications. This is followed by a briefing on the existing literature regarding adjunctive therapies and ongoing trials. Finally, we discuss the global response to the coronavirus disease-2019 (COVID-19) pandemic and the lessons learned for future pandemics.

TIMELINE TO PANDEMIC

Though the specific date varies according to different reports, it is postulated that the outbreak started in Wuhan around December 12, 2019, when multiple patients presented with similar clinical symptoms including fever, cough, dyspnea, and atypical pneumonia (3). On December 29, four cases of "pneumonia of unknown etiology" were officially reported by local hospitals using a surveillance mechanism that was established following the 2002–2003 SARS epidemic with the aim of allowing timely identification of novel pathogens. All four of these cases were thought to have a connection to a local seafood market, Huanan Seafood Market, which sold live non-aquatic wild animals (5, 6).

In an attempt to identify the causative pathogen, three bronchoalveolar lavage fluid samples from one patient with "pneumonia of unknown etiology" were collected and sent for identification on December 30. Whole genome sequencing and bioinformatic analyses revealed that the virus features were typical of the beta-coronavirus 2B lineage of the coronavirus (7). Additionally, the genome of the novel virus was found to be 96% identical to the bat SARS-like coronavirus strain BatCov RaTG13, a bat coronavirus detected in Rhinolophus affinis from Yunnan province (2).

On December 31, the Chinese authorities alerted the World Health Organization (WHO) of these cases. Due to the continued connection of emerging cases to the Huanan Seafood Market, the market was eventually closed on January 1, 2020 for sanitization. On January 6, the Chinese Center for Disease Control and Prevention (China CDC) activated a Level 2 emergency response. On January 8, a novel coronavirus was officially announced to be the cause of the outbreak and on January 10, the first genome sequence for the virus was released by China CDC. The novel virus was initially called the 2019 novel coronavirus (2019-nCoV). The WHO subsequently changed the name to SARS-CoV-2 on February 11 due to its vast resemblance to SARS-CoV (8).

The first case reported outside of China was on January 13 in Thailand. China CDC upgraded the emergency response to Level 1 on January 15 (9). On January 20, the CDC confirmed the first case in the United States (U.S.) in Washington state, which was linked to recent travel from Wuhan (10). Due to the continued surge of new cases, the Chinese government ordered a complete lock down of Wuhan on January 23. By January 30, the WHO declared a global health emergency and COVID-19 was declared a pandemic on March 11, 2020 (9) (**Figure 1**).

As of the beginning of June, there were more than 7 million confirmed cases of COVID-19 with more than 400 thousand deaths globally. This pandemic has spread to more than 200 countries, areas, or territories across the world (11). In comparison, SARS-CoV spread to 12 countries including the U.S. with a total of 8,096 confirmed cases and 774 deaths until it was contained in 2003 (12). MERS-CoV spread to 27 countries, including the U.S., with a total of 2,494 confirmed cases and 858 deaths (13) (**Table 1**).

THE ORIGIN OF SARS-COV-2

It is crucial to identify the origin, hosts, and evolutionary pathway of the causative pathogen of a pandemic to be able to implement proper control measures and help prevent future pandemics. Unfortunately, the exact origin of SARS-CoV-2 remains unclear so many theories have been proposed based on information stemming from SARS-CoV.

After the SARS epidemic in 2002, bats were first recognized to be hosts for coronaviruses and interest grew in identifying other potential mammal hosts (15). The majority of early cases of SARS occurred in patients with close contact to animals including market palm civets. Soon afterwards, SARS-CoV was cultivated from caged Himalayan palm civets from live wild markets in Guangdong, China. Upon further investigation, with the discovery of many coronaviruses phylogenetically related to SARS-CoV in bats from different provinces in China and other countries, bats were believed to be the natural reservoir for SARS-CoV, and the palm civet was a possible intermediate host. It was likely that the virus acquired multiple mutations in the market palm civets before spillover to humans (1, 6). Bats were also believed to be the natural reservoir for MERS-CoV and dromedary camels were thought to be the intermediate hosts. Bats have since been discovered to be the hosts of a minimum of 30 coronaviruses with available complete genome sequences (15). This may be an underestimation since many more coronaviruses may exist that have yet to be identified or sequenced.

As previously stated, SARS-CoV-2 has been found to be 96% identical at the whole genome level to the bat SARS-like coronavirus strain BatCov RaTG13, making it likely that bats served as reservoir hosts. With many theories not supportive of direct spillover from bats to humans, further investigation was conducted. Pangolins were then reported as potential intermediate hosts after samples were analyzed from Malytan pangolins, an endangered species illegally trafficked into southern China for use in old-fashioned Chinese medicine and as a food source. These were obtained from Guangdong and Guangxi, China during an anti-smuggling operation. Samples from the pangolins showed new coronavirus genomes with 85.5–92.4% resemblance to SARS-CoV-2. More remarkable was the 97.4% amino acid similarity in the receptor binding domain (RBD) of coronavirus genomes from pangolins compared to SARS-CoV-2. In comparison, the Bat CoV RaTG only had 89.2% amino acid similarity in the RBD with SARS-CoV-2. Up until

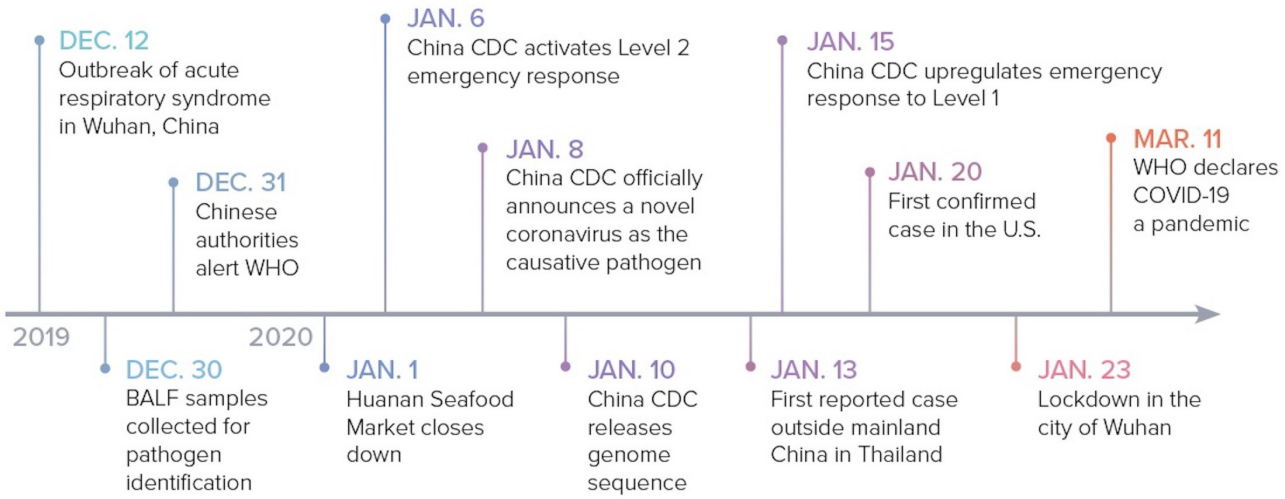


FIGURE 1 | COVID-19: timeline to pandemic. The sequence of events from the outbreak in Wuhan, China to the declaration of the COVID-19 pandemic. BALF, bronchoalveolar lavage fluid.

TABLE 1 | Comparison between SARS-CoV-2, MERS-CoV, and SARS-CoV.

	SARS-CoV-2	MERS-CoV	SARS-CoV
Pandemic/ epidemic year	2019-Present	2012	2002–2003
Coronavirus subfamily	Beta–Coronavirus	Beta–Coronavirus	Beta–Coronavirus
Natural reservoir	Bat	Bat	Bat
Intermediate host	Pangolin	Dromedary camel	Palm civets
Origin	Wuhan, China	Arabian Peninsula	Guangdong, China
Country spread	>180	27	26
Total cases to date	>7,000,000	2,494	8,096
Total deaths to date	>400,000	858	774
Total cases in the U.S. to date	>1,900,000	2	27
Case fatality rate*	1–7.2%	34.4%	9.6%

** Case fatality rate varies in different countries depending on different testing strategies, definition of COVID-19 related deaths, and population age. Numbers are subject to change with the ongoing COVID-19 pandemic (1, 11–14).*

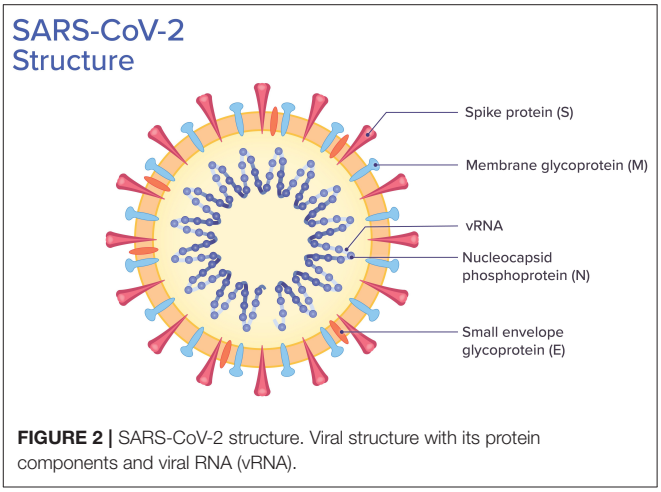


FIGURE 2 | SARS-CoV-2 structure. Viral structure with its protein components and viral RNA (vRNA).

now, bats and pangolins are the only two mammals known to be infected by SARS-CoV-2-related coronaviruses (6, 16) (**Table 1**).

VIRAL MORPHOLOGY

Coronaviruses are enveloped, positive single-stranded RNAs with the largest known RNA genome ranging from 26 to 32 kilobases in length (8, 17). They are spherical virions with a core shell and a surface that resembles a solar corona based on its surface protein projections, hence their name (Latin: corona = crown) (8). There are four main subfamilies; alpha-, beta-, gamma- and delta- coronaviruses.

Alpha- and beta-coronaviruses originate from mammals, mainly bats, and are thought to cause more severe and fatal diseases in humans, while gamma- and delta-viruses mainly originate from birds and pigs and are thought to cause asymptomatic or mild disease in humans (8).

SARS-CoV-2 belongs to the beta-coronavirus group, which also includes MERS-CoV and SARS-CoV. The latter shares ~75–80% of its viral genome with SARS-CoV-2 (8, 18). Beta-coronaviruses have three important envelope proteins: Spike (S) protein, Membrane (M) protein, and Envelope (E) protein. S protein mediates viral attachment to the cell membrane receptor, membrane fusion, and ultimately viral entry into the host cell. M protein, the most abundant membrane protein, together with E protein are responsible for the coronavirus membrane structure. Another component of the beta-coronavirus is the N protein, which is the protein component of the helical nucleocapsid that includes the genome RNA (19) (**Figure 2**).

MODE OF TRANSMISSION

According to current evidence, the WHO reports that SARS-CoV-2 transmission occurs via respiratory droplets and contact routes. Droplet transmission occurs through direct contact when a person is exposed to infective respiratory droplets when they are within 1 m of someone with respiratory symptoms including coughing and sneezing. Being within this distance puts the individual at risk of having their mucous membranes, including their mouth, nose and eyes, exposed to the droplets. Transmission can also occur through indirect contact by way of fomites on surfaces in the immediate environment around the infected person. Airborne transmission may be possible when aerosol-generating procedures are performed including endotracheal intubation, cardiopulmonary resuscitation, administration of nebulized treatments, and others (20).

Transmission of the virus can occur in the pre-symptomatic incubation period. A study in a nursing home showed that more than half of the residents with positive test results for SARS-CoV-2 infection were pre-symptomatic and most likely contributed to transmission (21). Asymptomatic transmission (i.e., in patients who never develop symptoms) can also occur as suggested in some studies (22, 23).

In terms of infectivity, the basic reproductive number (R0), which is defined as the expected average number of additional infectious cases that one infectious case can generate, was thought to range from 2.2 to 2.7 for SARS-CoV-2 infection in the early stages of the epidemic in China. This means that one person infected with SARS-CoV-2 can spread the infection to ∼2.2–2.7 people (5, 24). This number is subject to change with the progression of this pandemic, especially following the introduction of better control measures (5). The R0 for SARS-CoV was estimated to be around 3 after critically comparing various independent studies (25). However, the SARS-CoV outbreak was better controlled compared to the current pandemic due to successful isolation of infected patients (5). The R0 for MERS-CoV was estimated to range from 2 to 5 in Saudi Arabia and South Korea (26).

PATHOGENESIS

Although the pathogenesis of SARS-CoV-2 is not clearly understood, information regarding viral replication and pathogenesis can be extracted from what we know about other beta-coronaviruses (SARS-CoV and MERS-CoV) due to their similarities to SARS-CoV-2.

Direct Viral Injury

SARS-CoV-2 binds to epithelial cells in the oral and nasal cavities and can also migrate further down the respiratory tract into the conducting airways. SARS-CoV has been shown to infect primary ciliated cells in the conducting airway and therefore, it has been hypothesized that the same occurs with SARS-CoV-2. About 80% of the infected patients will have a mild course limited to the upper and conducting airways (27).

The virus can progress even further and can infect the alveolar type II pneumocyte cells, similar to SARS-CoV. It has been shown that SARS-CoV are released in large numbers from infected type II pneumocytes and cause cell apoptosis. Type II pneumocyte cells normally comprise 10–15% of total lung cells. They produce surfactant, which is responsible for the maintenance of surface tension in alveolar walls. These cells are also responsible for maintaining the lung epithelium after injury through epithelial regeneration (28). Therefore, as replicated viral particles are released from the cell and move on to infect more type II pneumocytes, the resulting apoptosis eventually causes diffuse alveolar damage and impaired gas exchange, which is hypothesized to lead to acute respiratory distress syndrome (ARDS). A similar mechanism is postulated for SARS-CoV-2 (27) (**Figure 3**).

Viral Replication Cycle

SARS-CoV-2 has been shown to use the angiotensin-converting enzyme 2 (ACE2) receptor for cell entry, similar to SARS-CoV (2). Through the examination of human tissue specimens, ACE2 receptors have been found in various organs and cells including the nasopharynx, nasal and oral mucosa, small intestine, colon, kidney, liver, vascular endothelium, and epithelial cells of lung alveoli (mainly type II pneumocytes) (29).

The RBD in the S protein of SARS-CoV-2 specifically recognizes its host ACE2 receptor. The viral RBD region is made of 394 glutamine residues and is recognized by 31 lysine residues of the human ACE2 receptor. Previous studies revealed that host susceptibility to SARS-CoV infection is mainly determined by the affinity between the host ACE2 receptor and the viral RBD in the early viral attachment phase. It is thought that this mechanism is likely similar in SARS-CoV-2 and that a genetic recombination event in the RBD region of SARS-CoV-2 may be the cause of its higher transmission rate as compared to SARS-CoV (30).

After cell entry, the viral RNA positive sense genome is released into the cell cytoplasm and undergoes translation and replication forming progeny genomes and sub-genomic mRNAs. The latter translates into membrane proteins, N protein, and a variety of accessory proteins (19). SARS-CoV has its own central enzyme called the RNA-dependent RNA polymerase, which, along with other viral and cellular proteins, composes the main replication complex responsible for replicating the viral genome (31).

The formed membrane proteins (S, M, and E) are then inserted into the rough endoplasmic reticulum (RER) and are transported to the endoplasmic reticulum-golgi intermediate compartment (ERGIC). N proteins along with genomic RNA then form nucleocapsids, which fuse into the ERGIC. Finally, the pathogen gets transported to the plasma membrane and is exported out of the cell via exocytosis (19, 32) (**Figure 3**).

Immune System Activation and Cytokine Storm Syndrome

When the virus enters the cell, its antigen is presented by the antigen-presenting cells (APCs) such as dendritic cells and macrophages. This leads to the activation of the body's

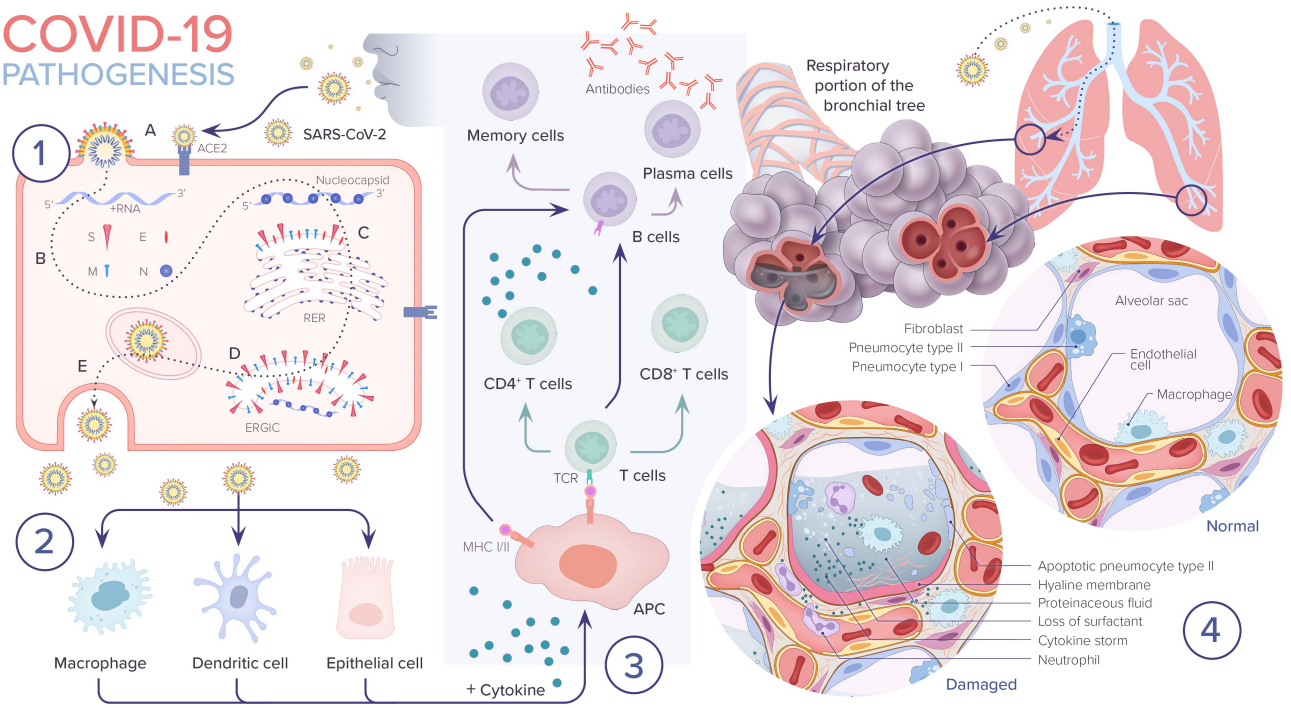


FIGURE 3 | COVID-19 pathogenesis. 1. A. SARS-CoV-2 enters the epithelial cell either via endocytosis or by membrane fusion through binding to ACE2 receptor and releasing its RNA into the cytoplasm. B. Viral RNA uses the cell's machinery to translate its viral non-structural and structural proteins and replicate its RNA. C. Viral structural proteins S, E, and M assemble in the rough endoplasmic reticulum (RER). D. Viral structures and nucleocapsid subsequently assemble in the endoplasmic reticulum golgi intermediate (ERGIC). E. New virion packed in golgi vesicles fuse with the plasma membrane and get released via exocytosis. 2. SARS-CoV-2 infection induces inflammatory factors that lead to activation of macrophages and dendritic cells. 3. Antigen presentation of SARS-CoV-2 via major histocompatibility complexes I and II (MHC I and II) stimulates humoral and cellular immunity resulting in cytokine and antibody production. 4. In severe COVID-19 cases, the virus reaches the lower respiratory tract and infects type II pneumocytes leading to apoptosis and loss of surfactant. The influx of macrophages and neutrophils induces a cytokine storm. Leaky capillaries lead to alveolar edema. Hyaline membrane is formed. All of these pathological changes result in alveolar damage and collapse, impairing gas exchange.

humoral and cellular immunities, which are mediated by virus-specific B and T cells (32, 33). Antigen presentation occurs via major histocompatibility complexes (MHC; or human leukocyte antigen (HLA) in humans) present on the surface of APCs and recognized by virus-specific cytotoxic T lymphocytes (CTLs). There are two major classes of MHCs involved in antigen presentation: MHC 1 and MHC II. SARS-CoV mainly depends on MHC I molecules. Unfortunately, the evidence regarding antigen presentation in SARS-CoV-2 is lacking and most of the information is extrapolated from prior studies done on SARS-CoV and MERS-CoV. Studies have shown that different HLA genotypes may be responsible for differences in host susceptibility to the virus and therefore, severity of disease. Patients infected with SARS-CoV with HLA-B*46:01 genotypes were shown to have more severe disease compared to those with different genotypes. This has not been clinically validated in studies on SARS-CoV-2 as of yet (32).

Once CD4+ T cells, also known as helper T cells, are activated, they cause the release of cytokines and chemokines (**Figure 3**). If exaggerated, this leads to the development of cytokine storm syndrome. The exact mechanism by which the immune system response to a viral infection can lead to cytokine storm syndrome is not completely understood. It has been shown that certain viruses are capable of altering the immune response to infection predisposing the host to develop a cytokine storm. Cytokine storm syndrome has been described in prior viruses including SARS-CoV, dengue and influenza virus. It remains a challenge to understand why some patients develop a cytokine storm while others do not. Research has shown that genetic polymorphisms, for example changes in the toll-like receptors (TLR), may play an important role in affecting host responses to certain infections, ultimately leading some to develop a cytokine storm (34).

Acute lung injury, including its severe form ARDS, is a common consequence of cytokine storm syndrome. This has been shown to occur in patients with SARS-CoV-2 infection with the development of diffuse lung injury, inflammation, and fluid buildup, which can ultimately lead to death. ARDS is also a common immunopathological event in both SARS-CoV and MERS-CoV (32). A study done in Wuhan, China noted that patients infected with SARS-CoV-2 had high amounts of pro-inflammatory cytokines and chemokines in their plasma. Critically ill patients who required intensive care unit (ICU) admission were found to have higher concentrations of cytokines in their plasma as compared to those with milder illness,

suggesting that cytokine storm was connected to disease severity (35). Similarly, patients with severe MERS-CoV and SARS-CoV infections showed higher levels of interleukin-6 (IL-6), a pro-inflammatory cytokine, and chemokines in their serum compared to those with mild disease (32).

IL-6 has received special attention. IL-6 plays a key role in cytokine storm syndrome. It has both anti-inflammatory and pro-inflammatory effects. IL-6 binds to its transmembrane and soluble receptors, which result in the activation of the inflammatory response potentially leading to cytokine storm (36). IL-6 levels have been shown to be ∼2.9 folds higher in patients with complicated disease, mainly those requiring ICU admission, compared to those with mild disease, with higher levels associated with a higher incidence of death (37).

Immunity

Individuals who become infected with SARS-CoV-2 produce antibodies against the virus. Most studies show that patients infected with SARS-CoV-2 develop antibody titers at days 10 to 15 after symptom onset. Based on preliminary evidence, these antibodies may have a protective role, however this is yet to be established (38, 39) An observational cohort study in Hong Kong showed a correlation between antibody titers detected by ELISA and virus neutralization titers (38). However, another study involving 175 patients who recovered from SARS-CoV-2 showed that a proportion of them developed very low antibody titers (below the detectable level) despite recovering from the disease. Therefore, further studies are needed to establish if antibody titers determine the likelihood to recover from disease (39). Further studies are needed to understand whether antibody titers reflect immunity, and if so, at what level and for how long.

RAAS Inhibitors and COVID-19

SARS-CoV and SARS-CoV-2 are involved with the renin-angiotensin-aldosterone system (RAAS) through ACE2, the enzyme that functions as a receptor for both viruses and also physiologically counters RAAS activation (40). Within RAAS, angiotensin I is converted to angiotensin II by ACE. Angiotensin II mediates vasoconstrictive and pro-inflammatory effects through angiotensin II type 1 receptor (AT_1R). ACE2, on the other hand, converts angiotensin II to angiotensin I-7, which binds to Mas receptor and facilitates numerous functions including vasodilation and anti-inflammatory effects. ACE2 also converts angiotensin I to angiotensin I-9, which can be further converted by ACE to angiotensin I-7. ACE2 limits the adverse vasoconstrictor and pro-inflammatory properties of angiotensin II by degrading it and by the formation of angiotensin I-7, counteracting its action. ACE inhibitors (ACE-Is) block the conversion of angiotensin I to angiotensin II. Angiotensin receptor blockers (ARBs) inhibit the binding of angiotensin II to AT_1R and angiotensin II type 2 receptor (AT_2R); its affinity for AT_1R, the main pathway by which angiotensin II exerts its pro-inflammatory effects, is 1,000 times greater than AT_2R (41) (**Figure 4**).

A large proportion of COVID-19 patients have preexisting hypertension. Also, patients with more severe illness are more likely to have hypertension than those with mild illness. This has sparked concerns that the RAAS inhibitors used for medical management of hypertension may somehow be contributing to poor outcomes through their effect on ACE2 (40). Conflicting data exists regarding the effect of RAAS inhibitors on levels and expression of ACE2 in various human tissues (41). In studies involving patients with a variety of cardiac conditions, plasma ACE2 activity was not higher among patients taking ACE-Is or ARBs when compared to patients not treated with these medications. On the other hand, in a longitudinal cohort study in Japan involving patients with hypertension, those who received long-term treatment with the ARB olmesartan had higher urinary ACE2 levels than control patients. However, the same findings were not present among patients using ARBs other than olmesartan or the ACE-I enalapril (40). Data showing the effects of RAAS inhibitors on lung-specific expression of ACE2 specifically are lacking (42).

It is unclear whether RAAS inhibitors increase, decrease, or have no effect on levels and expression of ACE2. It is also uncertain whether increased ACE2 would have protective or detrimental effects. It is thought that increased ACE2 would be detrimental as it would facilitate greater entry of SARS-CoV-2 into the cell causing higher disease virulence. Instead, some postulate that increased ACE2 may have a beneficial role in SARS-CoV-2 infection by attenuating virus-induced lung injury due to the vasodilator and anti-inflammatory role of the ACE2 pathway (42). Finally, others have proposed that SARS-CoV-2 entry into the cell downregulates ACE2 expression based on studies done *in vitro* in cultured cells, which showed that viral infection and replication contributed to reduced membrane ACE2. Down-regulation of ACE2 activity may be detrimental as it would cause unopposed accumulation of angiotensin II leading to the organ injury seen in COVID-19 (40).

The uncertainties outlined above make it difficult to offer guidance regarding the use of these medications in patients with COVID-19. The results of a retrospective Chinese study in Wuhan involving 1,178 patients hospitalized with COVID-19 showed that the frequency of severe disease, ARDS, and mortality did not differ in those using ACE-Is or ARBs compared to those not using these medications (43). Also, there is clear potential for harm associated with the withdrawal of RAAS inhibitors in patients in otherwise stable condition. RAAS inhibitors have well-established benefits in protecting the myocardium, and their withdrawal causes clinical decompensation in high-risk patients as has been shown in multiple studies. For example, in the Quinapril Heart Failure Trial, withdrawal of quinapril in patients with chronic symptomatic heart failure resulted in a progressive decline in clinical status. Among patients dealing with an unstable clinical status and ongoing myocardial injury due to COVID-19, withdrawal of RAAS inhibitors may pose an even higher risk (40).

Therefore, societies including the American College of Cardiology (ACC) have supported the continuation of RAAS inhibitors in patients in otherwise stable condition who are at risk for, are being evaluated for, or have been diagnosed with COVID-19 (40). The ACC advises that patients should continue taking RAAS inhibitors for conditions such as heart failure,

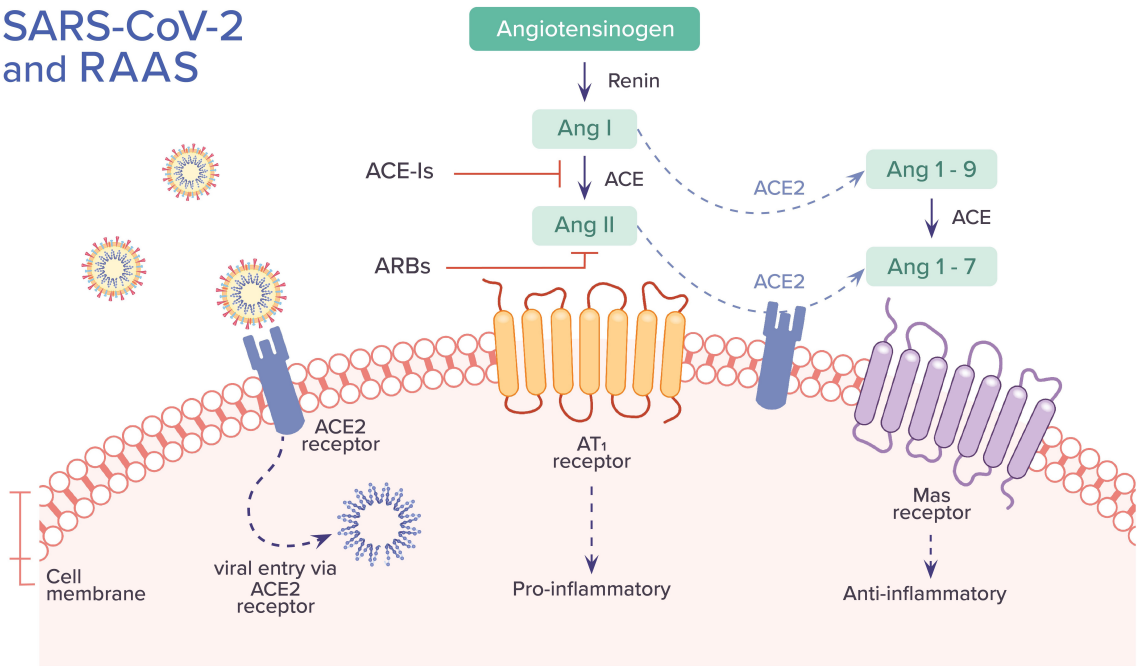


FIGURE 4 | The association between SARS-CoV-2 and the Renin-Angiotensin-Aldosterone System (RAAS). SARS-CoV-2 binds to ACE2 receptor and enters into the cell. It has been hypothesized that this process leads to down-regulation of surface ACE2, resulting in unopposed angiotensin II buildup and activity, leading to a pro-inflammatory cascade. Alternative hypotheses are further described in the text. The uncertainties regarding the role of ACE-Is and ARBs in COVID-19 are also discussed. Ang, angiotensin; ACE, angiotensin-converting enzyme; AT1, angiotensin II type 1 receptor.

hypertension, or ischemic heart disease, and that if COVID-19 occurs, "individualized treatment decisions should be made according to each patient's hemodynamic status and clinical presentation" (44).

HISTOPATHOLOGY

Compared to the robust clinical literature, there are relatively few published reports on the histopathology of COVID-19, none of which are large series (45–52). Published reports as of the time of this writing (April 25, 2020) are summarized in **Table 2**. The first description of COVID-19 histopathology came from China and consisted of a single case report based on post-mortem core biopsies of the lung, liver and heart (52). This was followed by another publication from China on the pathologic findings in two lobectomies for lung cancer in which the patients developed symptoms of COVID-19 after surgery (49). The authors postulated that the (rather non-specific) findings observed in the lungs possibly represented early COVID-19 pathology. On April 10, 2020, the first findings of complete autopsies in the English literature were described by Barton et al. from the United States (46).

This publication was followed by a few small autopsy series (including "limited autopsies") and another small series of post-mortem biopsies from China (48, 50, 51).

Thus, far, the most consistently reported finding in COVID-19 has been diffuse alveolar damage (DAD) in the lungs (**Figure 5A**). This finding has been observed in virtually every published case report or series thus far [**Table 2**; (46–52)]. DAD is a pathologic manifestation of severe acute lung injury. It is characterized by the presence of hyaline membranes in the acute stage and interstitial edema and fibroblast proliferation in the organizing stage. We would like to emphasize that DAD is not specific for COVID-19 but has a large list of potential causes, including shock, sepsis, severe trauma, other infections, connective tissue disease, drug toxicity, and toxic inhalants, among others (53–56). A subset of cases is idiopathic (57). Common secondary pathologic findings in DAD (regardless of etiology) include large, prominent and sometimes atypical type II pneumocytes, squamous metaplasia, and occasional thrombi within small pulmonary arteries. This last point is worth stressing: thrombi in the lung are well-known as a common secondary finding in DAD. They are thought to result from endothelial damage, which is central to the pathogenesis of DAD regardless of etiology. We stress this point to prevent misinterpretation of occasional thrombi in small arteries in the lung in the context of DAD as evidence of a more generalized thrombotic tendency. In fact, prominent thrombi were reported in lungs infected by H1N1, and at the time it was suggested that this finding might be unique to H1N1 (58).

Inflammatory infiltrates of various types have also been reported in COVID-19, including lymphocytic infiltrates in the airways and interstitium (46, 49), and neutrophils (45). An example of interstitial lymphocytic inflammatory infiltrates in the lung in a COVID-19 case is shown in **Figure 5B**.

TABLE 2 | Histopathology of COVID-19 in peer-reviewed English language journals.

Date	First author (country)	Specimen type	No. of cases	Main findings	DAD	Thrombi
Feb 18, 2020	Xu Z (China) (52)	Post-mortem biopsies of lung, liver, heart	1	DAD	Yes	None mentioned
Feb 28, 2020	Tian S (China) (49)	Lobectomies	2	Edema, intra-alveolar fibrin, mononuclear inflammatory cells	Yes ("early DAD pattern" in 1 of 2)	None mentioned
April 10, 2020	Barton LM (USA) (46)	Complete autopsies	2	DAD, chronic airway inflammation	Yes (1 case)	Few (lung, 1 case)
April 11, 2020	Karami P (Iran) (47)	"Autopsy of lungs"	1	Hyaline membranes, viral cytopathic effect	Yes (hyaline membrane noted)	None mentioned
April 14, 2020	Tian S (China) (50)	Post-mortem biopsies of lung, liver, heart	4	DAD	Yes	None mentioned
April 15, 2020	Magro C (USA) (48)	Limited autopsies (2), skin biopsies (3)	5	"Hemorrhagic pneumonitis" (lung), "thrombogenic vasculopathy" (skin)	Yes (hyaline membranes in 1 of 2 cases in which lungs were examined)	Yes (skin)
April 16, 2020	Barnes BJ (USA) (45)	Autopsies (brief mention)	3	"Neutrophil extracellular traps"	Not mentioned	None mentioned
April 20, 2020	Varga Z (Switzerland) (51)	Autopsies (2), small intestine resection (1)	3	Endotheliitis, DAD, viral inclusions in endothelial cells in kidney	Yes	"Only scattered fibrin thrombi"

DAD, Diffuse alveolar damage.

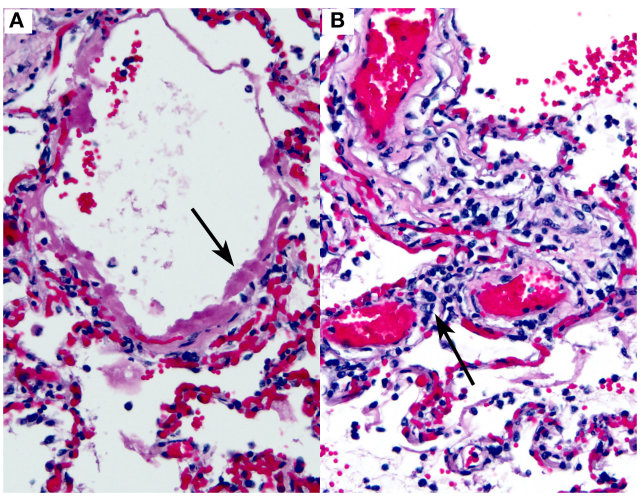


FIGURE 5 | COVID-19 lung autopsy specimen. This figure is original and based on data from (46). It demonstrates COVID-19 pathology as seen in the lungs of an autopsied case (case 1, Barton et al.). **(A)** Diffuse alveolar damage. The arrow points to a hyaline membrane. **(B)** Interstitial lymphocytic inflammatory infiltrate. The arrow indicates lymphocytes within an alveolar septum. Hematoxylin-eosin stain, 200×, both images.

There has been intense clinical interest around the development of thrombi in a subset of patients with COVID-19. Interestingly, a widespread thrombotic process has not been documented in the majority of pathology specimens examined thus far (**Figure 6** and **Table 2**). Only one report has illustrated a few thrombi in skin biopsies from three patients who presented with a purpuric rash. No published pathology reports have illustrated widespread multi-organ thrombi in the setting of COVID-19. In future pathology studies, it will be interesting to determine whether the clinical suspicion of widespread "microthrombosis" is confirmed by histopathology, and if so, to determine whether this is common or occurs only in a small subset of cases.

Other pathologic findings reported only sporadically in COVID-19 include viral inclusions, edema, intra-alveolar fibrin, and endotheliitis. To our knowledge, there are no reports of histologically documented myocarditis clearly attributable to COVID-19 thus far. Additionally, there is no evidence that any of the pathologic findings discussed above are pathognomonic of COVID-19.

CLINICAL SYMPTOMS

Clinical symptoms have been shown to occur most commonly between days 4 and 5 from exposure; however, studies have shown that the incubation period can last up to 14 days (5, 59). The most common symptoms reported in the literature so far include fever, cough, fatigue and shortness of breath, which are similar to other viral infections including the seasonal flu. One study identified 24 critically ill patients from nine Seattle-area hospitals with laboratory-confirmed COVID-19 infection with symptoms beginning 7 ± 4 days before admission. The most commonly reported symptoms were cough and shortness of breath and around 50% of patients had fever on admission (60). A case series study in New York, the epicenter of the pandemic in the U.S., that included 5,700 patients with COVID-19 infection found that 30.7% of the patients were febrile on

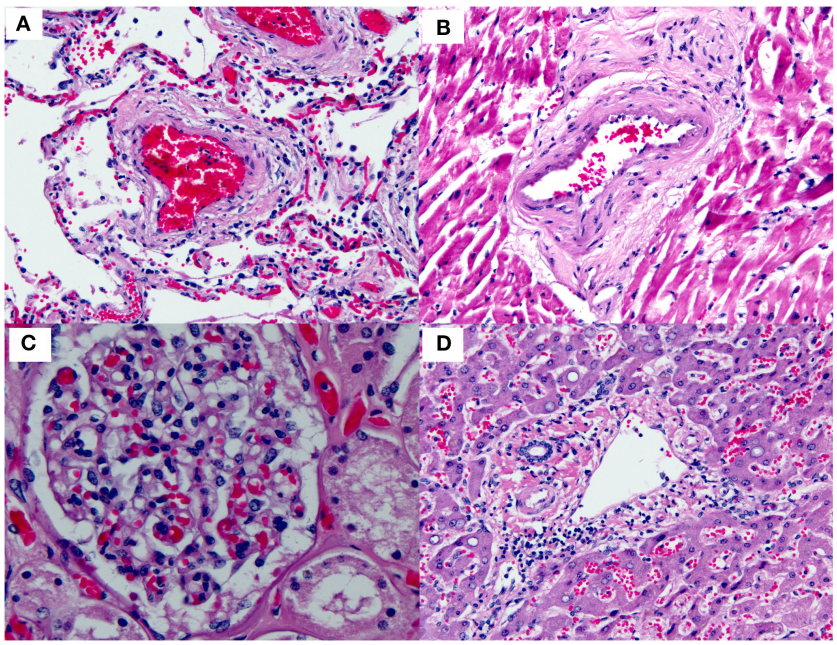


FIGURE 6 | Small blood vessels in various organs in COVID-19. This figure is original and based on data from (46). No thrombi are seen in the small blood vessels of the **(A)** Lung. **(B)** Heart. **(C)** Kidney (glomerulus). **(D)** Liver (portal tract) (autopsy case 1, Barton et al.).

TABLE 3 | A list of the most common clinical symptoms of SARS-CoV-2 infection based on a 1,099 patient study in China (59).

Symptoms	Percentage (%)
Fever	88.7
Cough	67.8
Fatigue	38.1
Sputum production	33.7
Shortness of breath	18.7
Myalgia or arthralgia	14.9
Sore throat	13.9
Headache	13.6
Chills	11.5
Nausea or vomiting	5
Nasal congestion	4.8
Diarrhea	3.8

admission (61). Another large study in China that extracted data from 1,099 patients with laboratory-confirmed COVID-19 showed that 43.8% of the patients had a fever on admission while 88.7% of patients developed a fever during their hospital stay. The second most commonly reported symptom was cough (67.8%) while fewer patients reported gastrointestinal symptoms such as nausea (5%) and diarrhea (3.8%) (59) (**Table 3**).

Anosmia and dysgeusia have also been reported in patients with SARS-CoV-2 infection. A cross-sectional survey study found that these symptoms were frequently reported in patients infected with SARS-CoV-2 and, in most cases, preceded the onset of other symptoms (62). Asymptomatic infection has also been discussed in the literature; however, the frequency remains unclear. A study of 55 asymptomatic carriers with confirmed SARS-CoV-2 infection on admission found that the majority of these patients ended up having mild symptoms and a mild disease course while asymptomatic infection was rare and was mainly in young patients between 18 and 29 years of age (63). Another study involving 634 patients infected with COVID-19 on a cruise ship in Japan found that 17.9% were asymptomatic (64).

DIAGNOSIS

SARS-CoV-2 RNA is detected via reverse-transcription polymerase chain reaction (RT-PCR) most commonly collected from nasopharyngeal (NP) swabs. In the United States, the CDC recommends the collection of NP swabs for asymptomatic individuals. Instead, specimens from symptomatic patients should be collected from bilateral anterior nares and mid-turbinate. An oropharyngeal (OP) swab could be collected if an NP swab is not possible. The CDC also recommends collecting sputum in patients with a productive cough, however sputum induction is not recommended. Also, when clinically indicted (i.e., patients who are mechanically intubated), a lower respiratory tract sample via a bronchioalveolar lavage (BAL) should be collected (65).

The accuracy of SARS-CoV-2 testing is yet to be established. It has been noted that RT-PCR testing for SARS-CoV-2 could be falsely negative either due to insufficient viral load if the specimen is collected too early or too late in the disease course, or due to technical errors like being handled or shipped improperly

(64, 66). There have been cases reported of patients presenting with classic computed tomography (CT) chest findings (bilateral peripheral distribution with multifocal lower lung involvement) combined with high clinical suspicion for SARS-CoV-2 infection who test negative on RT-PCR (67). Lower respiratory tract samples (i.e., BAL) are more likely to yield a positive result compared to upper respiratory tract samples. In a study involving 205 patients, 93% of BAL specimens (14 out of 15) were positive compared to 72% of NP swab specimens (72 out of 104) (68). Consequently, if initial testing is negative but clinical suspicion remains high, the WHO recommends repeat testing, preferably from a lower respiratory tract specimen, if possible.

Given that SARS-CoV-2 is a newly discovered virus, the antibody response in COVID-19 patients remains largely unknown. As of now, RT-PCR-based viral RNA is the current reference standard diagnostic tool for COVID-19 infections, but several studies are suggesting the incorporation of serologic antibody testing to aid in diagnosis of COVID-19 infections. These can be particularly useful in suspected patients with negative RT-PCR-based viral RNA and those with asymptomatic infections. In addition, these tests may improve the sensitivity of COVID-19 pathogenic diagnosis when combined with RT–PCR-based viral RNA testing.

In a study conducted by Zhao et al., among 173 patients with SARS-CoV-2 infection, the median seroconversion time for total antibodies, immunoglobulin-M (IgM), and immunoglobulin-G (IgG) against SARS-CoV-2 were day-11, day-12 and day-14, respectively. The presence of antibodies was <40% among patients within 1-week since onset, and rapidly increased to 100.0% for total antibodies, 94.3% for IgM, and 79.8% for IgG on day 15 after onset. In comparison, RNA detectability decreased from 66.7% in samples collected before day 7–45.5% during days 15–39 (69).

Another study by Long et al. showed that among 285 patients with COVID-19 infections, 100% of patients tested positive for antiviral IgG within 19 days after symptom onset. Within the same study, 4 out of 52 suspected cases with negative RT-PCR-based viral RNA for SARS-CoV-2 tested positive for virus-specific IgG or IgM (70).

Rapid point-of-care testing for SARS-CoV-2, which is also an IgG/IgM based test with a time to result of 20 min has shown a good specificity of 88.9% but low sensitivity of 36.4% making it a less effective test for screening (71). Despite their aid in diagnosis, antibody tests do impose limitations, especially as single screening tools since the sensitivity and specificity of serologic antibody tests are highly variable. Also, it might take several days from the onset of infection for the body to formulate these antibodies.

Similar to SARS-CoV and MERS-CoV, SARS-CoV-2 has been detected in blood and stool. Therefore, blood and stool specimens could be tested to aid with the diagnosis (66).

LABORATORY FINDINGS

Hospitalized patients with SAR-CoV-2 infection have been found to have varying white blood cell counts. A study by Huang et al. showed leukopenia ($< 4 \times 10^9$ per L) in 25% of patients, normal leukocyte counts ($4–10 \times 10^9$ per L) in 45% of patients, and leukocytosis ($>10 \times 10^9$ per L) in 30% of patients. Lymphopenia ($< 1 \times 10^9$ per L) was found in 63% of patients (35). Another study by Guan et al. showed that leukopenia was present in 33.7% of patients on admission and 36.2% of the cases had thrombocytopenia (59). In a systematic review and meta-analysis of 43 studies involving 3,600 patients, the most common laboratory abnormalities included elevated C-reactive protein (68.6%), lymphopenia (57.4%), and elevated lactate dehydrogenase (LDH) (51.6%) (72). A study done by Zhou et al. showed that elevated levels of LDH, serum ferritin, IL-6, and high sensitivity cardiac troponin I were all associated with worsening illness and higher mortality (73).

One of the most common laboratory findings in hospitalized patients with COVID-19 is an increased d-dimer level. In a large retrospective analysis study of 1,099 patients with confirmed COVID-19 in China, patients with more severe illness were more likely to have an elevated d- dimer level compared to patients with non-severe illness (59). In another retrospective analysis study of 183 patients with confirmed COVID-19 pneumonia in Wahun, non-survivors were found to have significantly higher d-dimer and fibrin degradation product (FDP) levels, and longer prothrombin time (PT) on admission compared to survivors. Fibrinogen and antithrombin (AT) levels were also significantly lower in non-survivors. Also, 71.4% of non-survivors had overt disseminated intravascular coagulation (DIC) during their hospitalization compared to only 0.6% of survivors. The results imply that abnormal coagulation parameters during COVID-19 pneumonia were significantly associated with poor prognosis (74). Studies also showed that blood urea nitrogen and creatinine levels progressively increased in critically ill patients (75).

RADIOLOGICAL FINDINGS

Chest CT abnormalities during the early stages of COVID-19 are usually peripheral and focal or multifocal ground-glass opacities affecting both lungs in ∼50–75% of patients (**Figure 7**). As the disease progresses, crazy paving and consolidation become the dominant CT findings, peaking around 9–13 days followed by slow clearing at ∼1 month and beyond. Up to 50% of patients with COVID-19 infection may have normal chest CT scans 0–2 days after the onset of symptoms (76). On the other hand, it has been shown that abnormal chest CT findings may develop in asymptomatic patients (77). In one study, chest CT images from patients with SARS-CoV-2 who were admitted to the hospital showed some level of abnormality in all patients and bilateral lung involvement in around 98% of patients (40 out of 41) (35). Another study showed 86.2% of chest CT images on COVID-19 positive patients were abnormal and only 17.9% of patients had normal chest CT images, all of whom had mild disease (59).

During pandemics, physicians rely more on portable chest x-ray (CXR) since it is widely available and creates less exposure risk for staff compared to CT. However, some studies have shown that CXR may lack sensitivity for the detection of some

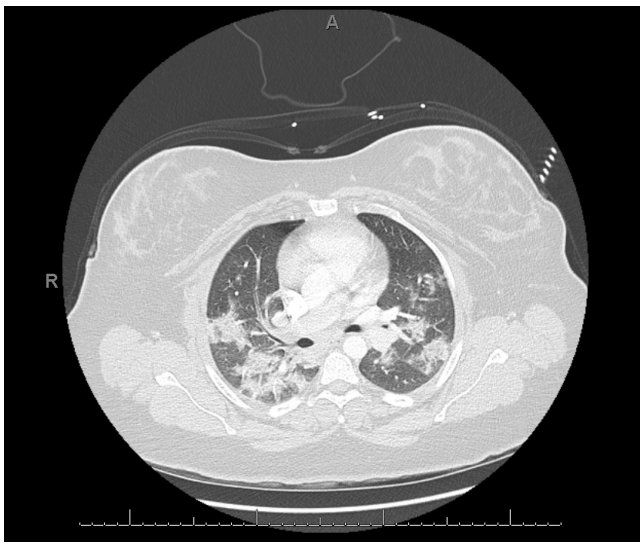


FIGURE 7 | COVID-19 positive patient chest computed tomography (CT). This figure is original and illustrates the findings from (76). It demonstrates bilateral, predominately peripheral, patchy ground-glass opacities consistent with multi-lobar pneumonia.

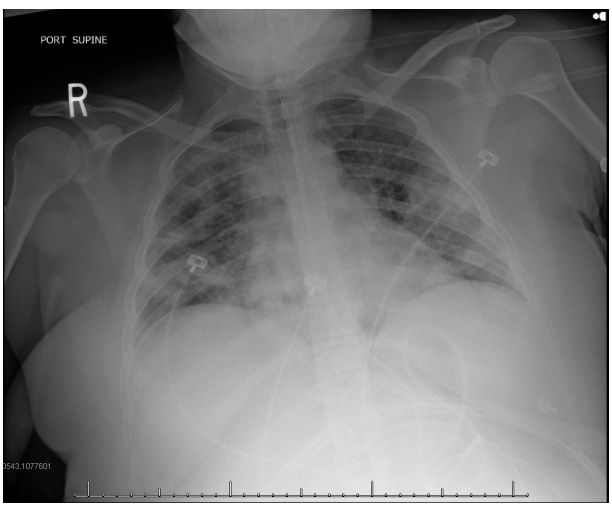


FIGURE 8 | COVID-19 positive patient chest x-ray (CXR). This figure is original and illustrates the findings from (78). It demonstrates bilateral predominately mid to lower lung field airspace opacities.

lung changes frequently seen in COVID-19, which are otherwise detected with CT. Similar to CT, the most common reported CXR findings in COVID-19 include ground-glass opacities and lung consolidation (**Figure 8**) (78).

SPECTRUM OF ILLNESS SEVERITY AND COURSE OF DISEASE

The spectrum of illness associated with SARS-CoV-2 infection ranges from mild to severe and even fatal infection. The largest study to date was done by China CDC, which included around 44,672 patients with confirmed SARS-CoV-2 infection. This study showed that among 44,415 patients, the majority of the cases (81%) were classified as mild disease (i.e., mild pneumonia or no pneumonia) while ∼14% were classified as severe disease (i.e., dyspnea with respiratory rate ≥30/min, blood oxygen saturation ≤93%, partial pressure of arterial oxygen to fraction of inspired oxygen ratio <300, and/or the development of diffuse lung infiltrates involving more than 50% of the lungs within 24–48 h), and 5% as critical disease (i.e., respiratory failure, shock, and/or multi-organ failure) (79).

The majority of patients with SARS-CoV-2 infection have been found to start with mild symptoms and, during the course of a week, progress to moderate or severe disease. A study done in Wuhan showed that, in the majority of patients, the median time to the development of dyspnea was 5 days, to hospital admission was 7 days, and to the development of ARDS was 8 days from the start of illness (75). Another study showed that the median time to mechanical ventilation was around 14.5 days from the onset of illness (73).

DISEASE COMPLICATIONS

ARDS is one of the major complications of SARS-CoV-2 infection. A study involving 138 patients in Wuhan, China showed that 19.6% of the patients developed ARDS. Other common complications identified in this study included shock (8.7%), arrhythmia (16.7%), and acute cardiac injury (7.2%) (59). Patients who were admitted and received care in the ICU were more likely to develop these complications than non-ICU patients (75).

Another study including 191 patients in Wuhan, China showed that the most common complication was sepsis (59%) followed by respiratory failure (54%), ARDS (31%), heart failure (23%), and septic shock (20%). Other less frequent complications included coagulopathy (19%), defined as 5-s extension of activated partial thromboplastin time or 3-s extension of prothrombin time, and acute cardiac injury (17%), defined as elevated high sensitivity cardiac troponin I to above the 99th percentile of the upper reference limit or new EKG and/or echocardiogram findings. Non-survivors suffered more of these complications compared to survivors (73).

Interestingly, cardiac events such as new or worsening congestive heart failure, myocardial infarctions, arrythmias, and cardiac arrest occurred more frequently in patients with associated pneumonia (73).

In severe COVID-19 disease, hypercoagulability can be stimulated by endothelial cell dysfunction, increased blood viscosity from hypoxia, or hypoxia-induced transcription factor-dependent signaling pathway (80, 81). Acute venous thromboembolism (VTE) has been reported in patients with SARS-CoV-2 infection. A Dutch study involving 184 ICU with proven COVID-19 found a 31% incidence of thrombotic complications, of which 27% comprised of radiographically confirmed VTE. Pulmonary embolism (PE) was the most frequent of these thrombotic complications (82). Another study in Wuhan, China showed that 66 out of the 143 hospitalized

patients with COVID-19 included in the study developed a lower extremity deep vein thrombosis (DVT). Their analysis suggested multifactorial causes of DVT in these patients including older age, more severe illness, more chronic illness, stasis, and high thrombotic and inflammatory abnormalities (83). A case report by Danzi et al. described the case of a 75-year-old COVID-19 positive hospitalized female radiographically diagnosed with a pulmonary embolism who had no other predisposing factors other than the acute infection with COVID-19 (84).

RISK FACTORS ASSOCIATED WITH SEVERE DISEASE

Many studies have shown that severe illness and death occur in patients with certain risk factors including older age and underlying medical comorbidities. A study done by Wu et al. showed that among 44,672 cases of COVID-19 in Wuhan, China, the majority of patients were 30 to 79 years of age (87%) followed by those aged 80 years and older (3%) while only 1% were aged 9 years and younger (79). Older age was one of the identified risk factors associated with poor prognosis and death (73). A study by Guan et al. showed that those with severe disease were older by a mean of 7 years compared to those with mild disease (59).

It remains unclear whether gender is an independent risk factor for more severe disease. A retrospective case series done in New York, showed that among the 393 patients with confirmed COVID-19, 60.6% were males. Also, males were more likely to receive mechanical ventilation (85). However, this correlation does not imply causation since this study did not adjust for other medical comorbidities.

A study by Guan et al. showed that patients with severe disease were more likely to have an underlying coexisting illness compared to those with non-severe disease (38.7 vs. 21%) (59). Another study done in Wuhan, China showed that among 191 patients with COVID-19, hypertension (30%) was the most commonly reported comorbidity followed by diabetes (19%), coronary heart disease (8%), and chronic obstructive lung disease (3%) (73). According to data from the CDC in the US, among 7,162 patients with reported medical problems, diabetes mellitus (10.9%), chronic lung disease (9.2%), and cardiovascular disease (9.0%) were the most commonly reported comorbidities. Immunocompromising conditions (3.7%) and chronic kidney disease (3%) were also reported (86). In a case series study in New York including 5,700 patients with COVID-19 infection, the most common comorbidities in hospitalized patients were hypertension (56.6%), obesity (41.7%), and diabetes (33.8%) (61). Obesity was found to be risk factor for intubation in a retrospective cohort study of 124 patients with SARS-CoV-2 infection. Of the patients who were intubated, 47.6% had a body mass index (BMI) > 30 kg/m^2 and 28.2% had a BMI > 35 kg/m^2 (87).

CASE FATALITY RATES

Case Fatality Rates (CFR) is defined as the ratio between confirmed deaths and confirmed cases. To date, SARS-CoV-2 seems to have a lower CFR compared to SARS-CoV and MERS-CoV (**Table 1**).

Estimating the CFR in an ongoing pandemic can be challenging since it is subject to considerable change as more cases emerge and more outcomes unveil. CFR varies depending on multiple factors including testing strategies. For example, low testing capability can lead to an over-estimation of the CFR by causing an under-estimation of the number of confirmed cases (14, 88).

China CDC estimated the CFR to be around 2.3% among 44,672 confirmed COVID-19 cases, 8% of whom were aged 70–79 years, 14.8% aged 80 years and older, and 0% were among those aged 9 years or younger (79).

According to the Italian National Institute of Health, the CFR in Italy was 7.2% among 22,512 cases up to March 17, 2020 (14). According to data collected by the South Korea CDC, the CFR was 1.79% among 10,237 cases up to April 5, 2020 (89). According to the CDC in the United States, the CFR was 2.5% among 304,826 cases as of April 5, 2020 (65).

The numbers of cases and deaths are evolving on a daily basis; however, it remains unclear why there is such a big difference in CFR across different countries. As noted, the overall CFR in Italy is significantly higher than that reported in China (2.3 vs. 7.2%). The demographic characteristics of the Italian population in 2019 showed that ∼23% of its population was above the age of 65. This might somehow explain Italy's higher CFR compared to other countries affected by the virus with smaller proportions of their populations in this age group.

However, when data was stratified according to age groups, the CFR in Italy and China were similar among those aged 0–69 years but the CFR remained significantly higher in Italy compared to China in patients aged 70 years and older (14). Understanding this significant difference in CFR across countries remains challenging and further studies are required to comprehend it fully.

INVESTIGATIONAL APPROACHES AND ADJUNCTIVE THERAPIES

Unfortunately, up until this point, there has yet to be a vaccine or proven effective therapy against SARS-CoV-2 infection. While many trials, including much needed randomized controlled trials (RCTs), are currently underway, the mainstay of therapy remains supportive care. This ranges from symptomatic treatment to ventilator support for patients with ARDS depending on illness severity. This also includes recognizing and treating superimposed bacterial infections and/or sepsis early on. Many of the current clinical trials are investigating drugs that were previously used to treat SARS-CoV and MERS-CoV. These will be discussed further below.

Chloroquine/Hydroxychloroquine

Chloroquine and hydroxychloroquine are widely used anti-malarial drugs. Hydroxychloroquine is a chloroquine analog with less drug to drug interaction and a better safety profile.

Both chloroquine and hydroxychloroquine are shown to inhibit the growth of SARS-CoV-2 *in vitro* and decrease viral replication in a concentration-dependent manner. Hydroxychloroquine was found to be more potent. It has been hypothesized that both chloroquine and hydroxychloroquine may inhibit SARS-CoV-2 replication. They may do this by changing the pH at the surface of the cell membrane thereby inhibiting fusion in addition to inhibiting nucleic acid replication, glycosylation, and viral assembly and release (90).

Multicenter clinical trials in China showed that chloroquine was effective and had an acceptable safety profile in patients with SARS-CoV-2 pneumonia (91). Hydroxychloroquine is currently under investigation in various RCTs in the Unites States for treatment in patients with SARS-CoV-2 infection and also for pre-exposure and post-exposure prophylaxis. In one retrospective cohort study involving 1,438 patients hospitalized in metropolitan New York, treatment with hydroxychloroquine, azithromycin, or both was not associated with significantly lower in-hospital mortality when compared to neither treatment. However, the interpretation of these findings may be limited by the observational design (92).

In another randomized, double-blind, placebo-controlled trial in the US, 821 asymptomatic participants were randomly assigned to receive either placebo or hydroxychloroquine 4 days after exposure to someone with confirmed COVID-19. The study found that hydroxychloroquine did not prevent illness related to COVID-19 or confirmed infection when used as postexposure prophylaxis within this timeframe (93).

On June 15, 2020, the U.S. Food and Drug Administration (FDA) revoked the emergency use authorization granted on March 28, 2020 for chloroquine phosphate and hydroxychloroquine sulfate in certain hospitalized COVID-19 patients. They cite the serious cardiac adverse events and other protentional serious side effects to outweigh the potential benefits of their use (94).

Azithromycin

Azithromycin is a macrolide antibiotic that has been widely used in patients with chronic pulmonary inflammatory disorders and/or community acquired pneumonia for its anti-inflammatory effect (95). However, there is limited data suggesting the beneficial effect of azithromycin in combination with chloroquine/hydroxychloroquine in the treatment of ARDS in patients with SARS-CoV-2 infection.

An open-label non-randomized clinical trial of 36 patients done in China showed a synergistic effect combining hydroxychloroquine and azithromycin in treatment of SARS-CoV-2 infection by reducing the detection of SARS-CoV-2 RNA in specimens from the upper respiratory tract (96). However, this study did not comment on the clinical benefit of this combination. Another small observational study in China showed that combining hydroxychloroquine and azithromycin for the treatment of SARS-CoV-2 in hospitalized patients had no clinical benefit and no evidence of rapid viral RNA clearance (97). Hydroxychloroquine and azithromycin can both lead to corrected QT (QTc) prolongation, which can lead to fatal arrythmias. Therefore, they should be used with caution in patients with prolonged QTc and those with certain medical conditions such as hepatic or renal disease.

Remdesivir

Remdesivir is a novel nucleotide analog that incorporates into nascent viral RNA chains and causes premature termination inhibiting viral replication. Remdesivir has been shown to be an effective antiviral agent against beta-coronaviruses such as SARS-CoV and SARS-MERS in mice, non-human primates and *in vitro*, and is currently in clinical trials for the treatment of Ebola virus (98).

A study in China showed that remdesivir is highly effective in controlling SARS-CoV-2 infection *in vitro* (98). Another study that was recently published involving compassionate-use of remdesivir showed clinical improvement in 68% of patient (36 out of 53) who had severe SARS-CoV-2 infection; 57% were extubated and 47% were discharged (99).

Despite its promising results *in vitro*, *in vivo* in animal models, and in compassionate-use studies in humans, remdesivir is still not approved by the FDA for use as a standard of care therapy due to lack of established data on safety and efficacy in humans. The biopharmaceutical company Giliad has initiated two phase 3 clinical trials to evaluate the safety and efficacy of this drug in COVID-19 patients.

Lopinavir-Ritonavir

Lopinavir-ritonavir is a protease inhibitor combination that has been used against human immunodeficiency virus (HIV) infection. This drug was proven to have *in vitro* activity against SARS-CoV; however, it does not seem to have a clear benefit during the current outbreak (100). A randomized, controlled, open-label trial that included 199 patients assessed the use of lopinavir–ritonavir treatment in patients with SARS-CoV-2 and showed no benefit with administration of the drug compared to standard care alone, which comprised of antibiotics, vasopressors, renal replacement therapy, extracorporeal membrane oxygenation (ECMO) and/or supplemental oxygen/invasive ventilation if needed. Gastrointestinal adverse events were higher in the lopinavir–ritonavir group compared to those receiving standard-care alone; however, adverse events were higher in the standard-care group overall (101).

Favipiravir

Favipiravir is an RNA polymerase inhibitor that is used for the treatment of influenza in China. Favipiravir is able to block the replication of RNA viruses by blocking the RNA-dependent RNA polymerase (RdRp) enzyme. Therefore, favipiravir may have antiviral activity against SARS-CoV-2, which is also an RNA virus (102). Clinic trials involving the use of this drug in treating SARS-CoV-2 infection are currently ongoing.

IL-6 Pathway Inhibitors

As previously mentioned, cytokine storm syndrome and increased levels of IL-6 have been described in patients with

severe SARS-CoV-2 infection. IL-6 levels were found to be 2.9-fold higher in patients with severe complicated SARS-CoV-2 infection, including those with ARDS, when compared to mild, non-complicated disease. Until now, there are no RCTs showing that IL-6 inhibitors benefit patients with SARS-CoV-2 infection. However, preliminary investigation demonstrated that IL-6 inhibitors are safe and efficacious in these patients. A single non-randomized, single-arm study showed that patients with severe SARS-CoV-2 infection who received tocilizumab, an IL-6 inhibitor, showed significant clinical improvement including decreased oxygen requirement and resolution of radiographic abnormalities (37).

Treatment guidelines from China's National Health Commission included tocilizumab for patients with severe SARS-CoV-2 infection who also have increased IL-6 levels based on a multicenter, randomized controlled trial (103). Multiple IL-6 inhibitors including tocilizumab, sarilumab, and siltuximab are currently under investigation in clinical trials in China.

Ivermectin

Ivermectin is an FDA-approved medication for the treatment of various parasites and has an established safety profile in humans. Ivermectin has been shown to inhibit *in vitro* replication of various positive single stranded RNA viruses such as dengue and west Nile (104, 105). This drug has recently demonstrated *in vitro* activity against SARS-CoV-2 when a single dose was able to control viral replication within 24–48 h. It is hypothesized that this is likely through the inhibition of importin α/β1 heterodimer, which mediates nuclear import of viral proteins, a process that many RNA viruses rely on during infection (105, 106). The FDA has not yet approved ivermectin for the prevention or treatment of SARS-CoV-2 infection. RCTs studying the efficacy and safety of this drug in COVID-19 are still lacking.

Corticosteroids

The use of glucocorticoids in patients with SARS-CoV-2 infection, especially in those with severe disease, was a point of major controversy. The rationale behind their use is to decrease lung inflammation as seen in ARDS. However, this comes with adverse effects such as inhibiting the immune response and thus increasing the risk of secondary infections as well as delaying viral clearance (107). A Cochran review published in July 2019 that included 48 RCTs found insufficient evidence to determine if corticosteroids were effective at reducing mortality and duration of mechanical ventilation in patients with ARDS (108).

A recent randomized, controlled, open label study known as the RECOVERY trial included 2,104 COVID-19 patients in the United Kingdom (UK) who were randomly allocated to receive 6 mg of dexamethasone per day for up to 10 days compared to standard of care therapy alone. Preliminary results from this trial showed that dexamethasone use reduced 28-days mortality among those with severe disease (i.e., those receiving invasive mechanical ventilation or oxygen support) but not among patients with mild disease (i.e., those who did not receive any respiratory support) (109).

Prior to this trial, many treatment guidelines stated that corticosteroids were either not recommended or contraindicated in COVID-19 patients. The WHO welcomed the preliminary results of the RECOVERY trial and will soon be updating their guidelines regarding how and when dexamethasone should be used in COVID-19 patients (110).

Convalescent Plasma

Convalescent plasma (CP) therapy is a classic adaptive immunotherapy that has been used for decades in the prevention and treatment of various diseases. CP was used in prior epidemics including SARS-CoV, MERS-CoV, and H1N1 in 2009 and it showed successful results with a safe profile (111). Given the similarity between SARS-CoV-2, SARS-CoV, and MERS-CoV, CP may have potential efficacy in this current pandemic. However, no RCTs involving CP in SARS-CoV-2 infection have been completed as of yet, and hence the risks and benefits remain unclear.

In an uncontrolled case series, the treatment of five patients with severe SARS-CoV-2 infection and ARDS with CP showed clinical improvement in all five cases. All of these patients showed stabilization in their vital signs, decrease in inflammatory biomarkers (CRP, IL-6 and procalcitonin), and improvement of abnormalities on imaging. Three out of five of these patients were successfully extubated (112). Another study showed that the use of CP in 10 patients with severe SARS-CoV-2 infection resulted in significant clinical improvement with no side effects. All patients had disappearance of viremia within 7 days, improvement in their clinical symptoms, and improvement in their chest radiographic abnormalities (111).

In the United States, the FDA is accommodating emergent investigational application for the use of CP in patients with severe or immediate life-threatening SARS-CoV-2 infection, such as those in respiratory failure, septic shock and/or multiorgan failure (113).

Heparin

As more studies emerge linking coagulopathies to COVID-19 including systemic thrombosis and DIC, this raises the question whether heparin should be used in hospitalized patients to prevent these complications.

In a retrospective study in China that included 449 patients, patients who received a prophylactic dose of heparin when they had sepsis-induced coagulopathy (SIC) score ≥ 6 and a d-dimer level >6-fold of upper limit of normal had decreased mortality (81). Based on the limited available data, the International Society of Thrombosis and Hemostasis (ISTH) recommends the measurement of d-dimer, PT, and platelet count for all patients with COVID-19 infection to help with risk stratification. The society also recommends the administration of low molecular weight heparin at prophylactic dose to all hospitalized patients with no contraindications (114). RCTs examining the use of heparin in COVID-19 patients are required to make appropriate recommendations.

Vitamin C

Vitamin C, also known as ascorbic acid, has antioxidant properties and plays a significant role in reducing inflammatory response. Studies have shown that ascorbic acid down-regulates

the production of pro-inflammatory cytokines (115). These concepts have generated interest in the use of ascorbic acid in the management of inflammatory conditions. In a recent randomized clinical trial involving 167 patients in the intensive care unit, intravenous infusion of high-dose ascorbic acid compared to placebo did not significantly reduce organ dysfunction scores or improve levels of biomarkers indicating inflammation among patients with sepsis and ARDS, two disease processes heavily associated with inflammation (116). A randomized controlled trial is currently underway and in phase 2 to study the clinical efficacy and safety of vitamin C infusion for treatment of COVID-19 pneumonia (117).

Zinc

It has been shown that increased zinc concentration inside the cell can effectively impair replication of a number of RNA viruses such as influenza and polioviruses. A study showed that zinc in combination with zinc-ionophores like pyrithione inhibited the replication of SARS-CoV in cell cultures (118). Therefore, zinc supplementation may be of potential benefit for prophylaxis and treatment of COVID-19 and it is currently under investigation in multiple clinical trials in combination with other agents including hydroxychloroquine, vitamin C, and vitamin D (119).

Montelukast

Montelukast has been shown to suppress oxidative stress and have anti-inflammatory effects. Use of high dose montelukast has been effective in the treatment of acute asthma. Because much of the morbidity and mortality from COVID-19 infection is due to excessive inflammatory processes, it is thought that montelukast may play a role in limiting the progression of disease (120). One of the protein complexes involved in cytokine production and inflammatory responses is NF-B (nuclear factor kappa-light-chain-enhancer of activated B cells). Therefore, inhibition of the NF-B signalizing pathway has been investigated for potential therapeutic options in inflammatory diseases. Montelukast inhibits the signaling of NF-B and other proinflammatory mediators. Its use in COVID-19 infection is currently being studied in a large clinical trial, which is in phase 3, compared with placebo (121).

Potential Vaccines

To date, there is no vaccine proven effective against SARS-CoV-2 infection. There are numerous potential vaccines currently being investigated. The COVID-19 vaccine research and development landscape includes 115 vaccine candidates globally as of April 8, 2020. 78 of these candidates are confirmed, 73 of which are at exploratory or preclinical stages (122). One of the more advanced candidates that has recently moved into clinical development in the United States involves a messenger RNA platform (mRNA-1273), which encodes for the viral S protein of SARS-CoV-2 (123).

COVID-19 RESPONSE

Many have criticized the global response to COVID-19 due to the rapidly increasing number of cases and deaths worldwide. It is important to highlight the sequence of events in this response in order to recognize areas of concern and associated consequences, and to extract potential lessons and improvements for future pandemics.

As previously mentioned, the cluster of cases identified in Wuhan were reported to the WHO by Chinese authorities on December 31, 2019 and confirmed to be associated with a novel coronavirus, later termed COVID-19, on January 8, 2020 (124). There have been multiple reports of suspected intimidation of clinicians who initially identified cases linked to COVID-19, which likely led to a delay in the release of information and a lack of transparency (125).

On January 17, consistent with existing communicable disease response protocols based on previous pandemics, the CDC introduced screening of travelers entering at 5 major US airports on direct and connecting flights from Wuhan, China. Travel bans were not instituted by the Chinese government until January 24, when they started restricting travel in and out of Hubei province (124). However, according to Wuhan officials, by the time these travel restrictions were instituted, 5 million people had already traveled from Wuhan to other locations for Lunar New Year (126). These restrictions were placed almost 1 month after the first cases of COVID-19 were detected. This delay in travel restrictions and continued ability of citizens traveling from high-risk areas to freely pass through international borders with minimal health screening allowed individuals potentially infected with COVID-19 to spread the infection both nationally and internationally (125).

As cases began to spread outside of Mainland China, on January 21, the CDC activated its Emergency Operations Center to optimize coordination for domestic and international COVID-19 response efforts. The WHO director-general declared that the COVID-19 outbreak constitutes a Public Health Emergency of International Concern (PHEIC) on January 30 (124). The International Health Regulations (IHR) grants the WHO director-general to declare a PHEIC for an extraordinary event that requires a coordinated international response as it poses a public health risk to other states through international spread. The WHO has previously declared five PHEICs: H1N1 in 2009, Polio in 2014, Ebola in West Africa in 2014, Zika in 2016, and Ebola in the Democratic Republic of Congo in 2019. This declaration is a powerful signal to the international community to launch a surge public health response and mobilize both political action and funding (126). This declaration acknowledging and widely broadcasting the severity of this outbreak came 1 month after the initial cluster of cases, possibly delaying appropriate containment measures (125).

Just 1 day later, on January 31, the secretary of the US Department of Health and Human Services (HHS) declared the response to COVID-19 a US public health emergency (124). This declaration authorizes enhanced federal powers, interjurisdictional coordination, additional resources, and waivers of specific regulations. The exercise of federal powers is based on the need to prevent dire public health, national security, economic, and societal consequences. The federal powers exercised by the HHS in the response to COVID-19 goes beyond those ever used for other public health emergencies

such as Ebola, SARS, and H1N1 influenza. Following this declaration, federal agencies immediately implemented travel warnings, border protections, and entry bans (127). Since this declaration, multiple federal agencies, the CDC, and state and local health departments have also implemented other aggressive measures in an attempt to slow the spread of this illness, better prepare the health care systems for widespread transmission with considerable associated illness, and gain a better understanding of COVID-19 to guide public health recommendations and the development of diagnostics, therapeutics, and vaccines (124).

Perhaps, the most apparent and life-changing measure to the general public is the implementation of mitigation strategies, which are non-pharmaceutical interventions for communities with local transmission. These strategies are based on lessons learned from previous pandemics and are interventions that assist in slowing transmission of the virus in communities. This is an especially important feat prior to the wide availability of a pandemic vaccine. These strategies include "personal protective measures for everyday use" like self-isolation and hand hygiene; "personal protective measures reserved for pandemics" like home quarantine and wearing face masks when ill; "community measures aimed at increasing social distancing" like closing schools and stopping mass gatherings; and "environmental measures" like cleaning all surfaces that are frequently touched (128). The timing of the implementation of these strategies during the current pandemic has been under scrutiny.

A SECOND WAVE

As seen in multiple previous pandemics including the influenza pandemic of 1918, the first wave is often followed several months later by a second wave of infections that could potentially be even worse than the first. A second wave can be caused by a region being re-exposed to infection by an influx of infected people from another. The degree of the resulting new outbreak will depend on the level of immunity in the first region from the initial wave. This will be influenced by multiple factors including the potential for endogenous loss of immunity in the first population and the introduction of people who are not immune, for example, individuals moving from one state to another in the U.S. (129). To date, mitigation strategies have been effective at controlling the pandemic in several regions. A study by Aleta et al. showed that removing these restrictions could lead to a second wave of COVID-19 infections that could overwhelm the health care system. However, combining this with enhanced testing and contact tracing can reduce transmission and allow for reopening of economic activities, while having a manageable impact on the health care system even in the absence of herd immunity (130).

LESSONS LEARNED FOR FUTURE PANDEMICS

As this pandemic continues to develop and continues to take the lives of so many, there are innumerable lessons to be learned for future pandemics. To begin with, it is crucial to establish clear whistleblowing policies for potential global health emergencies. This will allow for transparency and help encourage clinicians to bring important information to light as soon as they are detected. Once high-risk areas have been identified, precautions including travel restrictions and quarantines should be implemented as soon as a possible health threat is identified. Also, framework should be developed to escalate a threat status earlier for fast-spreading diseases (125). It is then crucial to implement population-based interventions including social distancing, quarantine, and isolation actions promptly. And finally, it is imperative for health care systems along with local, regional, and global forces to work together to ensure better preparedness for future pandemics in all aspects including staffing, supplies, the number of hospital beds, testing capacity, research and development, and policy. A high price was paid for these difficult lessons to be learned, so it is now our responsibility to dedicate the appropriate funding and efforts to prevent this level of catastrophe from repeating itself (131).

CONCLUSION

Pandemics propose an immense challenge to public health, health care systems, and global economic security. Due to modern agricultural practices that increase human-animal interface, new zoonotic coronaviruses are likely to continue to spillover from animals to humans causing future outbreaks. Gaining insight into every aspect of coronaviruses is crucial to implement proper control measures to help prevent these outbreaks or lessen their impact on humans and society if they were to still happen. Special focus should be placed on understanding their pathophysiology to help better tailor and generate effective drug therapies and vaccinations. Nevertheless, our ability to handle future outbreaks will rely on the actions we take based on the lessons we have learned from previous pandemics. We hope that the rapidly developing research on the current COVID-19 pandemic will help provide the new information needed to fill these gaps.

AUTHOR CONTRIBUTIONS

NC, SC, and RB are the first, second, and third authors, respectively. They contributed equally to the literature search and the writing for the whole manuscript. AA contributed to the literature search and the preliminary writing for radiologic findings. AS and MR edited and commented on the final manuscript. SM, ES, ED, and LB contributed to the literature search and the writing of the histopathology section, in addition to providing original histopathology figures. IH is the senior and corresponding author. She provided the idea, supervised the entire process from inception to the final submission, and edited the final manuscript.

ACKNOWLEDGMENTS

We would like to acknowledge Audrey Bell for her contribution with the illustrations in **Figures 1–4**.

REFERENCES

1. Cui J, Li F, Shi ZL. Origin and evolution of pathogenic coronaviruses. *Nat Rev Microbiol.* (2019) 17:181–92. doi: 10.1038/s41579-018-0118-9
2. Zhou P, Yang XL, Wang XG, Hu B, Zhang L, Zhang W, et al. A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature.* (2020) 579:270–3. doi: 10.1038/s41586-020-2012-7
3. Zhu N, Zhang D, Wang W, Li X, Yang B, Song J, et al. A novel coronavirus from patients with pneumonia in China, 2019. *N Engl J Med.* (2020) 382:727–33. doi: 10.1056/NEJMoa2001017
4. Andersen KG, Rambaut A, Lipkin WI, Holmes EC, Garry RF. The proximal origin of SARS-CoV-2. *Nat Med.* (2020) 26:450–2. doi: 10.1038/s41591-020-0820-9
5. Li Q, Guan X, Wu P, Wang X, Zhou L, Tong Y, et al. Early transmission dynamics in Wuhan, China, of novel coronavirus-infected pneumonia. *N Engl J Med.* (2020) 382:1199–207. doi: 10.1056/NEJMoa2001316
6. Zheng J. SARS-CoV-2: an emerging coronavirus that causes a global threat. *Int J Biol Sci.* (2020) 16:1678–85. doi: 10.7150/ijbs.45053
7. World Health Organization. *Report of the WHO-China joint mission on coronavirus disease 2019 (COVID-19).* (2020). Available online at: https://www.who.int/docs/default-source/coronaviruse/who-china-joint-mission-on-covid-19-final-report.pdf (accessed April 14, 2020).
8. Velavan TP, Meyer CG. The COVID-19 epidemic. *Trop Med Int Health.* (2020) 25:278–80. doi: 10.1111/tmi.13383
9. World Health Organization. *WHO Timeline - COVID-19.* (2020). Available online at: https://www.who.int/news-room/detail/08-04-2020-who-timeline---covid-19 (accessed April 20, 2020).
10. Holshue ML, Debolt C, Lindquist S, Lofy KH, Wiesman J, Bruce H, et al. First case of 2019 novel coronavirus in the United States. *N Engl J Med.* (2020) 382:929–36. doi: 10.1056/NEJMoa2001191
11. World Health Organization. *Coronavirus Disease (COVID-19) Pandemic.* (2020). Available online at: https://www.who.int/emergencies/diseases/novel-coronavirus-2019 (accessed June 11, 2020).
12. World Health Organization. *Summary of Probable SARS Cases With Onset of Illness From 1 November 2002 to 31 July 2003.* (2020). Available online at: https://www.who.int/csr/sars/country/table2004_04_21/en/ (accessed April 16, 2020).
13. World Health Organization. *Middle East Respiratory Syndrome Coronavirus (MERS-CoV).* (2020). Available online at: https://www.who.int/emergencies/mers-cov/en/ (accessed April 16, 2020).
14. Onder G, Rezza G, Brusaferro S. Case-fatality rate characteristics of patients dying in relation to COVID-19 in Italy. *JAMA.* (2020) 323:1775–6. doi: 10.1001/jama.2020.4683
15. Wong ACP, Li X, Lau SKP, Woo PCY. Global epidemiology of bat coronaviruses. *Viruses.* (2019) 11:174. doi: 10.3390/v11020174
16. Lam TT, Shum MH, Zhu HC, Tong YG, Ni XB, Liao YS, et al. Identifying SARS-CoV-2 related coronaviruses in Malayan pangolins. *Nature.* (2020) 583:282–5. doi: 10.1038/s41586-020-2169-0
17. Weiss SR, Leibowitz JL. Coronavirus pathogenesis. *Adv Virus Res.* (2011) 81:85–164. doi: 10.1016/B978-0-12-385885-6.00009-2
18. Perlman S. Another decade, another coronavirus. *N Engl J Med.* (2020) 382:760–2. doi: 10.1056/NEJMe2001126
19. Masters PS. The molecular biology of coronaviruses. *Adv Virus Res.* (2006) 66:193–292. doi: 10.1016/S0065-3527(06)66005-3
20. World Health Organization. *Modes of Transmission of Virus Causing COVID-19: Implications for IPC Precaution Recommendations.* (2020). Available online at: https://www.who.int/news-room/commentaries/detail/modes-of-transmission-of-virus-causing-covid-19-implications-for-ipc-precaution-recommendations (accessed June 11, 2020).
21. Arons MM, Hatfield KM, Reddy SC, Kimball A, James A, Jacobs JR, et al. Presymptomatic SARS-CoV-2 infections and transmission in a skilled nursing facility. *N Engl J Med.* (2020) 382:2081–90. doi: 10.1056/NEJMoa2008457
22. Pan X, Chen D, Xia Y, Wu X, Li T, Ou X, et al. Asymptomatic cases in a family cluster with SARS-CoV-2 infection. *Lancet Infect Dis.* (2020) 20:410–1. doi: 10.1016/S1473-3099(20)30114-6
23. Bai Y, Yao L, Wei T, Tian F, Jin DY, Chen LJ, et al. Presumed asymptomatic carrier transmission of COVID-19. *JAMA.* (2020) 323:1406–07. doi: 10.1001/jama.2020.2565
24. Sanche S, Lin YT, Xu C, Romero-Severson E, Hengartner N, Ke R. High contagiousness and rapid spread of severe acute respiratory syndrome coronavirus 2. *Emerg Infect Dis.* (2020) 26:1470–7. doi: 10.3201/eid2607.200282
25. Bauch CT, Lloyd-Smith JO, Coffee MP, Galvani AP. Dynamically modeling SARS and other newly emerging respiratory illnesses: past, present, and future. *Epidemiology.* (2005) 16:791–801. doi: 10.1097/01.ede.0000181633.80269.4c
26. Choi S, Jung E, Choi BY, Hur YJ, Ki M. High reproduction number of Middle East respiratory syndrome coronavirus in nosocomial outbreaks: mathematical modelling in Saudi Arabia and South Korea. *J Hosp Infect.* (2018) 99:162–8. doi: 10.1016/j.jhin.2017.09.017
27. Mason RJ. Pathogenesis of COVID-19 from a cell biology perspective. *Eur Respir J.* (2020) 55:2000607. doi: 10.1183/13993003.00607-2020
28. Mossel EC, Wang J, Jeffers S, Edeen KE, Wang S, Cosgrove GP, et al. SARS-CoV replicates in primary human alveolar type II cell cultures but not in type I-like cells. *Virology.* (2008) 372:127–35. doi: 10.1016/j.virol.2007.09.045
29. Hamming I, Timens W, Bulthuis ML, Lely AT, Navis G, Van Goor H. Tissue distribution of ACE2 protein, the functional receptor for SARS coronavirus. A first step in understanding SARS pathogenesis. *J Pathol.* (2004) 203:631–7. doi: 10.1002/path.1570
30. Shereen MA, Khan S, Kazmi A, Bashir N, Siddique R. COVID-19 infection: origin, transmission, and characteristics of human coronaviruses. *J Adv Res.* (2020) 24:91–8. doi: 10.1016/j.jare.2020.03.005
31. Cheng A, Zhang W, Xie Y, Jiang W, Arnold E, Sarafianos SG, et al. Expression, purification, and characterization of SARS coronavirus RNA polymerase. *Virology.* (2005) 335:165–76. doi: 10.1016/j.virol.2005.02.017
32. Li X, Geng M, Peng Y, Meng L, Lu S. Molecular immune pathogenesis and diagnosis of COVID-19. *J Pharm Anal.* (2020) 10:102–8. doi: 10.1016/j.jpha.2020.03.001
33. Li G, Fan Y, Lai Y, Han T, Li Z, Zhou P, et al. Coronavirus infections and immune responses. *J Med Virol.* (2020) 92:424–32. doi: 10.1002/jmv.25685
34. Tisoncik JR, Korth MJ, Simmons CP, Farrar J, Martin TR, Katze MG. Into the eye of the cytokine storm. *Microbiol Mol Biol Rev.* (2012) 76:16–32. doi: 10.1128/MMBR.05015-11
35. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet.* (2020) 395:497–506. doi: 10.1016/S0140-6736(20)30183-5
36. Zhang C, Wu Z, Li JW, Zhao H, and Wang GQ. Cytokine release syndrome in severe COVID-19: interleukin-6 receptor antagonist tocilizumab may be the key to reduce mortality. *Int J Antimicrob Agents.* (2020) 55:105954. doi: 10.1016/j.ijantimicag.2020.105954
37. Coomes EA, Haghbayan, H. Interleukin-6 in COVID-19: a systematic review and meta-analysis. *medRxiv [Preprint].* (2020). doi: 10.1101/2020.03.30.20048058
38. To KK, Tsang O T., Leung WS, Tam AR, Wu TC, Lung DC, et al. Temporal profiles of viral load in posterior oropharyngeal saliva samples and serum antibody responses during infection by SARS-CoV-2: an observational cohort study. *Lancet Infect Dis.* (2020) 20:565–74. doi: 10.1016/S1473-3099(20)30196-1
39. Wu F, Wang A, Liu M, Wang Q, Chen J, Xia S, et al. *Neutralizing Antibody Responses to SARS-CoV-2 in a COVID-19 Recovered Patient Cohort and Their Implications.* Preprint repository name (2020). Available online at: https://www.medrxiv.org/content/10.1101/2020.03.30.20047365v2 (accessed April 16, 2020).
40. Vaduganathan M, Vardeny O, Michel T, Mcmurray JJV, Pfeffer MA, Solomon SD. Renin-angiotensin-aldosterone system inhibitors in patients with COVID-19. *N Engl J Med.* (2020) 382:1653–9. doi: 10.1056/NEJMsr2005760
41. Sanchis-Gomar F, Lavie C, Perez-Quilis C, Henry B, Lippi G. Angiotensin-converting enzyme 2 and antihypertensives (angiotensin receptor blockers and angiotensin-converting enzyme inhibitors) in coronavirus disease 2019. *Mayo Clinic Proceedings.* (2020) 95:1222–30. doi: 10.1016/j.mayocp.2020.03.026

42. Patel AB, Verma A. COVID-19 angiotensin-converting enzyme inhibitors angiotensin receptor blockers: what is the evidence? *JAMA*. (2020) 323:1769–70. doi: 10.1001/jama.2020.4812
43. Li J, Wang X, Chen J, Zhang H, Deng A. Association of renin-angiotensin system inhibitors with severity or risk of death in patients with hypertension hospitalized for coronavirus disease 2019 (COVID-19) infection in Wuhan, China. *JAMA Cardiol*. (2020). doi: 10.1001/jamacardio.2020.1624. [Epub ahead of print].
44. Aronson JK, Ferner RE. Drugs and the renin-angiotensin system in covid-19. *BMJ*. (2020) 369:m1313. doi: 10.1136/bmj.m1313
45. Barnes BJ, Adrover JM, Baxter-Stoltzfus A, Borczuk A, Cools-Lartigue J, Crawford JM, et al. Targeting potential drivers of COVID-19: neutrophil extracellular traps. *J Exp Med*. (2020) 217:e20200652. doi: 10.1084/jem.20200652
46. Barton LM, Duval EJ, Stroberg E, Ghosh S, Mukhopadhyay S. COVID-19 autopsies, Oklahoma, USA. *Am J Clin Pathol*. (2020) 153:725–33. doi: 10.1093/ajcp/aqaa062
47. Karami P, Naghavi M, Feyzi A, Aghamohammadi M, Novin MS, Mobaien A, et al. Mortality of a pregnant patient diagnosed with COVID-19: a case report with clinical, radiological, and histopathological findings. *Travel Med Infect Dis*. (2020). doi: 10.1016/j.tmaid.2020.101665. [Epub ahead of print].
48. Magro C, Mulvey JJ, Berlin D, Nuovo G, Salvatore S, Harp J, et al. Complement associated microvascular injury and thrombosis in the pathogenesis of severe COVID-19 infection: a report of five cases. *Transl Res*. (2020) 220:1–13. doi: 10.1016/j.trsl.2020.04.007
49. Tian S, Hu W, Niu L, Liu H, Xu H, Xiao SY. Pulmonary pathology of early-phase 2019 novel coronavirus (COVID-19) pneumonia in two patients with lung acncer. *J Thorac Oncol*. (2020) 15:700–4. doi: 10.20944/preprints202002.0220.v2
50. Tian S, Xiong Y, Liu H, Niu L, Guo J, Liao M, et al. Pathological study of the 2019 novel coronavirus disease (COVID-19) through postmortem core biopsies. *Mod Pathol*. (2020) 33:1007–14. doi: 10.20944/preprints202003.0311.v1
51. Varga Z, Flammer AJ, Steiger P, Haberecker M, Andermatt R, Zinkernagel AS, et al. Endothelial cell infection and endotheliitis in COVID-19. *Lancet*. (2020) 395:1417–8. doi: 10.1016/S0140-6736(20)30937-5
52. Xu Z, Shi L, Wang Y, Zhang J, Huang L, Zhang C, et al. Pathological findings of COVID-19 associated with acute respiratory distress syndrome. *Lancet Respir Med*. (2020) 8:420–2. doi: 10.1016/S2213-2600(20)30076-X
53. Askin FB, Katzenstein AL. Pneumocystis infection masquerading as diffuse alveolar damage: a potential source of diagnostic error. *Chest*. (1981) 79:420–2. doi: 10.1378/chest.79.4.420
54. Parambil JG, Myers JL, Aubry MC, Ryu JH. Causes and prognosis of diffuse alveolar damage diagnosed on surgical lung biopsy. *Chest*. (2007) 132:50–7. doi: 10.1378/chest.07-0104
55. Mukhopadhyay S, Philip AT, Stoppacher R. Pathologic findings in novel influenza A (H1N1) virus ("Swine Flu") infection: contrasting clinical manifestations and lung pathology in two fatal cases. *Am J Clin Pathol*. (2010) 133:380–7. doi: 10.1309/AJCPXY17SULQKSWK
56. Mukhopadhyay S, Mehrad M, Dammert P, Arrossi AV, Sarda R, Brenner DS, et al. Lung biopsy findings in severe pulmonary illness associated with e-cigarette use (vaping). *Am J Clin Pathol*. (2020) 153:30–9. doi: 10.1093/ajcp/aqz182
57. Mukhopadhyay S, Parambil JG. Acute interstitial pneumonia (AIP): relationship to Hamman-Rich syndrome, diffuse alveolar damage (DAD), and acute respiratory distress syndrome (ARDS). *Semin Respir Crit Care Med*. (2012) 33:476–85. doi: 10.1055/s-0032-1325158
58. Harms PW, Schmidt LA, Smith LB, Newton DW, Pletneva MA, Walters LL, et al. Autopsy findings in eight patients with fatal H1N1 influenza. *Am J Clin Pathol*. (2010) 134:27–35. doi: 10.1309/AJCP35KOZSAVNQZW
59. Guan WJ, Ni ZY, Hu Y, Liang WH, Ou CQ, He JX, et al. Clinical characteristics of coronavirus disease 2019 in China. *N Engl J Med*. (2020) 382:1708–20. doi: 10.1056/NEJMoa2002032
60. Bhatraju PK, Ghassemieh BJ, Nichols M, Kim R, Jerome KR, Nalla AK, et al. Covid-19 in critically ill patients in the Seattle region - case series. *N Engl J Med*. (2020) 382:2012–22. doi: 10.1056/NEJMoa2004500
61. Richardson S, Hirsch JS, Narasimhan M, Crawford JM, Mcginn T, Davidson KW, et al. Presenting characteristics, comorbidities, outcomes among 5700 patients hospitalized with COVID-19 in the New York City area. *JAMA*. (2020) 323:2052–9. doi: 10.1001/jama.2020.6775
62. Giacomelli A, Pezzati L, Conti F, Bernacchia D, Siano M, Oreni L, et al. Self-reported olfactory and taste disorders in SARS-CoV-2 patients: a cross-sectional study. *Clin Infect Dis*. (2020). doi: 10.1093/cid/ciaa330. [Epub ahead of print].
63. Wang Y, Liu Y, Liu L, Wang X, Luo N, Ling L. Clinical outcome of 55 asymptomatic cases at the time of hospital admission infected with SARS-Coronavirus-2 in Shenzhen, China. *J Infect Dis*. (2020) 221:1770–4. doi: 10.1093/infdis/jiaa119
64. Mizumoto K, Kagaya K, Zarebski A, Chowell G. Estimating the asymptomatic proportion of coronavirus disease 2019 (COVID-19) cases on board the Diamond Princess cruise ship, Yokohama, Japan, 2020. *Euro Surveill*. (2020) 25:2000180. doi: 10.2807/1560-7917.ES.2020.25.10.2000180
65. Centers for Disease Control and Prevention. *Coronavirus Disease 2019 (COVID-19)*. (2020). Available online at: https://www.cdc.gov/coronavirus/2019-ncov/hcp/therapeutic-options.html (accessed April 13, 2020).
66. World Health Organization. *Laboratory Testing for Coronavirus Disease 2019 (COVID-19) In Suspected Human Cases: Interim Guidance, 19 March 2020*. (2020). Available online at: https://apps.who.int/iris/handle/10665/331329 (accessed April 13, 2020).
67. Xie X, Zhong Z, Zhao W, Zheng C, Wang F, Liu J. Chest CT for typical 2019-nCoV pneumonia: relationship to negative RT-PCR testing. *Radiology*. (2020). doi: 10.1148/radiol.2020200343. [Epub ahead of print].
68. Wang W, Xu Y, Gao R, Lu R, Han K, Wu G, et al. Detection of SARS-CoV-2 in different types of clinical specimens. *JAMA*. (2020) 323:1843–4. doi: 10.1001/jama.2020.3786
69. Zhao J, Yuan Q, Wang H, Liu W, Liao X, Su Y, et al. Antibody responses to SARS-CoV-2 in patients of novel coronavirus disease 2019. *Clin Infect Dis*. (2020). doi: 10.1093/cid/ciaa344. [Epub ahead of print].
70. Long QX, Liu BZ, Deng HJ, Wu GC, Deng K, Chen YK, et al. Antibody responses to SARS-CoV-2 in patients with COVID-19. *Nat Med*. (2020) 26:845–8. doi: 10.1038/s41591-020-0897-1
71. Dohla M, Boesecke C, Schulte B, Diegmann C, Sib E, Richter E, et al. Rapid poist-of-care testing for SARS-CoV-2 in a community screening setting shows low sensitivity. *Public Health*. (2020) 182:170–2. doi: 10.1016/j.puhe.2020.04.009
72. Fu L, Wang B, Yuan T, Chen X, Ao Y, Fitzpatrick T, et al. Clinical characteristics of coronavirus disease 2019 (COVID-19) in China: a systematic review and meta-analysis. *J Infect*. (2020) 80:656–5. doi: 10.1016/j.jinf.2020.03.041
73. Zhou F, Yu T, Du R, Fan G, Liu Y, Liu Z, et al. Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: a retrospective cohort study. *Lancet*. (2020) 395:1054–62. doi: 10.1016/S0140-6736(20)30566-3
74. Tang N, Li D, Wang X, Sun Z. Abnormal coagulation parameters are associated with poor prognosis in patients with novel coronavirus pneumonia. *J Thromb Haemost*. (2020) 18:844–7. doi: 10.1111/jth.14768
75. Wang D, Hu B, Hu C, Zhu F, Liu X, Zhang J, et al. Clinical characteristics of 138 hospitalized patients with 2019 novel coronavirus-infected pneumonia in Wuhan, China. *JAMA*. (2020) 323:1061–9. doi: 10.1001/jama.2020.1585
76. Kanne JP, Little BP, Chung JH, Elicker BM, Ketai LH. Essentials for radiologists on COVID-19: an update-radiology scientific expert panel. *Radiology*. (2020). doi: 10.1148/radiol.2020200527. [Epub ahead of print].
77. Shi H, Han X, Jiang N, Cao Y, Alwalid O, Gu J, et al. Radiological findings from 81 patients with COVID-19 pneumonia in Wuhan, China: a descriptive study. *Lancet Infect Dis*. (2020) 20:425–34. doi: 10.1016/S1473-3099(20)30086-4
78. Jacobi A, Chung M, Bernheim A, Eber C. Portable chest X-ray in coronavirus disease-19 (COVID-19): a pictorial review. *Clinical Imaging*. (2020) 64:35–42. doi: 10.1016/j.clinimag.2020.04.001
79. Wu Z, Mcgoogan JM. Characteristics of important lessons from the coronavirus disease 2019 (COVID-19) outbreak in China: summary of a report of 72314 cases from the Chinese Center for Disease Control Prevention. *JAMA*. (2020) 323:1239–42. doi: 10.1001/jama.2020.2648

80. Gupta N, Zhao YY, Evans CE. The stimulation of thrombosis by hypoxia. *Thromb Res.* (2019) 181:77–83. doi: 10.1016/j.thromres.2019.07.013
81. Tang N, Bai H, Chen X, Gong J, Li D, Sun Z. Anticoagulant treatment is associated with decreased mortality in severe coronavirus disease 2019 patients with coagulopathy. *J Thromb Haemost.* (2020) 18:1094–9. doi: 10.1111/jth.14817
82. Klok FA, Kruip JHA, van der Meer NJM, Arbous MS, Gommers DAMPJ, Kant KM. et al. Incidence of thrombotic complications in critically ill ICU patients with COVID-19. *Thrombosis Res.* (2020) 191:145–7. doi: 10.1016/j.thromres.2020.04.013
83. Zhang L, Feng X, Zhang D, Jiang C, Mei H, Wang J, et al. Deep vein thrombosis in hospitalized patients with coronavirus disease 2019 (COVID-19) in Wuhan, China: prevelance, risk factors, outcome. *Circulation AHA*. (2020) 142:114–128. doi: 10.1161/CIRCULATIONAHA.120.046702
84. Danzi GB, Loffi M, Galeazzi G, and Gherbesi EAcute pulmonary embolism COVID-19 pneumonia: a random association? *Eur Heart J.* (2020) 41:1858. doi: 10.1093/eurheartj/ehaa254
85. Goyal P, Choi JJ, Pinheiro LC, Schenck EJ, Chen R, Jabri A, et al. Clinical characteristics of Covid-19 in New York City. *N Engl J Med.* (2020) 382:2372–4. doi: 10.1056/NEJMc2010419
86. Chow N, Fleming-Dutra K, Gierke R, Hall A, Hughes M, Pilishyili T, et al. Preliminary estimates of the prevalence of selected underlying health conditions among patients with coronavirus disease 2019 – United States, February 12-March 28, 2020. *MMWR Morb Mortal Wkly Rep.* (2020) 69:382–6. doi: 10.15585/mmwr.mm6913e2
87. Simonnet A, Chetboun M, Poissy J, Raverdy V, Noulette J, Duhamel A, et al. High prevalence of obesity in severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) requiring invasive mechanical ventilation. *Obesity (Silver Spring).* (2020) 28:1195–99. doi: 10.1002/oby.22831
88. Ruan S. Likelihood of survival of coronavirus disease 2019. *Lancet Infect Dis.* (2020) 20:630–1. doi: 10.1016/S1473-3099(20)30257-7
89. KCDC. *The Updates on COVID-19 in Korea as of 5 April.* (2020). Available online at: https://www.cdc.go.kr/board/board.es?mid=a30402000000&bid=0030 (accessed April 5, 2020).
90. Yao X, Ye F, Zhang M, Cui C, Huang B, Niu P, et al. *In vitro* antiviral activity and projection of optimized dosing design of hydroxychloroquine for the treatment of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). *Clin Infect Dis.* (2020) ciaa237. doi: 10.1093/cid/ciaa237
91. Gao J, Tian Z, Yang X. Breakthrough: chloroquine phosphate has shown apparent efficacy in treatment of COVID-19 associated pneumonia in clinical studies. *Biosci Trends.* (2020) 14:72–3. doi: 10.5582/bst.2020.01047
92. Rosenberg ES, Dufort EM, Udo T, Wilberschied LA, Kumar J, Tesoriero J, et al. Association of treatment with hydroxychloroquine or azithromycin with in-hospital mortality in patients with COVID-19 in New York state. *JAMA*. (2020) 323:2493–502. doi: 10.1001/jama.2020.8630
93. Boulware DR, Pullen MF, Bangdiwala AS, Pastick KA, Lofgren SM, Okafor EC, et al. A randomized trial of hydroxychloroquine as postexposure prophylaxis for Covid-19. *N Engl J Med.* (2020) NEJMoa2016638. doi: 10.1056/NEJMoa,2016638
94. U. S., Food, and Drug Administration. *Coronavirus (COVID-19) Update: FDA Revokes Emergency Use Authorization for Chloroquine and Hydroxychloroquine.* (2020). Available online at: https://www.fda.gov/news-events/press-announcements/coronavirus-covid-19-update-fda-revokes-emergency-use-authorization-chloroquine-and (accessed June 15, 2020).
95. Amsden GW. Anti-inflammatory effects of macrolides–an underappreciated benefit in the treatment of community-acquired respiratory tract infections and chronic inflammatory pulmonary conditions? *J Antimicrob Chemother.* (2005) 55:10–21. doi: 10.1093/jac/dkh519
96. Gautret P, Lagier JC, Parola P, Hoang VT, Meddeb L, Mailhe M, et al. Hydroxychloroquine and azithromycin as a treatment of COVID-19: results of an open-label non-randomized clinical trial. *Int J Antimicrob Agents.* (2020). doi: 10.1016/j.ijantimicag.2020.105949. [Epub ahead of print].
97. Molina JM, Delaugerre C, Le Goff J, Mela-Lima B, Ponscarme D, Goldwirt L, et al. No evidence of rapid antiviral clearance or clinical benefit with the combination of hydroxychloroquine and azithromycin in patients with severe COVID-19 infection. *Med Mal Infect.* (2020) 50:348. doi: 10.1016/j.medmal.2020.03.006
98. Wang M, Cao R, Zhang L, Yang X, Liu J, Xu M, et al. Remdesivir and chloroquine effectively inhibit the recently emerged novel coronavirus (2019-nCoV) *in vitro*. *Cell Res.* (2020) 30:269–71. doi: 10.1038/s41422-020-0282-0
99. Grein J, Ohmagari N, Shin D, Diaz G, Asperges E, Castagna A, et al. Compassionate use of remdesivir for patients with severe Covid-19. *N Engl J Med.* (2020) 382:2327–36. doi: 10.1056/NEJMoa2007016
100. Groneberg DA, Poutanen SM, Low DE, Lode H, Welte T, Zabel P. Treatment and vaccines for severe acute respiratory syndrome. *Lancet Infect Dis.* (2005) 5:147–55. doi: 10.1016/S1473-3099(05)70022-0
101. Cao B, Wang Y, Wen D, Liu W, Wang J, Fan G, et al. A trial of lopinavir-ritonavir in adults hospitalized with severe Covid-19. *N Engl J Med.* (2020) 382:1787–99. doi: 10.1056/NEJMoa2001282
102. Dong L, Hu S, Gao J. Discovering drugs to treat coronavirus disease 2019 (COVID-19). *Drug Discov Ther.* (2020) 14:58–60. doi: 10.5582/ddt.2020.01012
103. Mehta P, Mcauley DF, Brown M, Sanchez E, Tattersall RS, Manson JJ, et al. COVID-19: consider cytokine storm syndromes and immunosuppression. *Lancet.* (2020) 395:1033–4. doi: 10.1016/S0140-6736(20)30628-0
104. Tay MYF, Fraser JE, Chan WKK, Moreland NJ, Rathore AP, Wang C, et al. Nuclear localization of dengue virus (DENV) 1-4 non-structural protein 5; protection against all 4 DENV serotypes by the inhibitor Ivermectin. *Antiviral Res.* (2013) 99:301–6. doi: 10.1016/j.antiviral.2013.06.002
105. Caly L, Druce JD, Catton MG, Jans DA, Wagstaff KM. The FDA-approved drug ivermectin inhibits the replication of SARS-CoV-2 *in vitro*. *Antiviral Res.* (2020) 178:104787. doi: 10.1016/j.antiviral.2020.104787
106. Yang SNY, Atkinson SC, Wang C, Lee A, Bogoyevitch MA, Borg NA, et al. The broad spectrum antiviral ivermectin targets the host nuclear transport importin alpha/beta1 heterodimer. *Antiviral Res.* (2020) 177:104760. doi: 10.1016/j.antiviral.2020.104760
107. Russell CD, Millar JE, Baillie JK. Clinical evidence does not support corticosteroid treatment for 2019-nCoV lung injury. *Lancet.* (2020) 395:473–5. doi: 10.1016/S0140-6736(20)30317-2
108. Lewis SR, Pritchard MW, Thomas CM, Smith AF. Pharmacological agents for adults with acute respiratory distress syndrome. *Cochrane Database Syst Rev.* (2019) 7:CD004477. doi: 10.1002/14651858.CD004477.pub3
109. Horby P, Lim WS, Emberson J, Mafham M, Bell J, Linsell L, et al. *Effect of Dexamethasone in Hospitalized Patients With COVID-19 – Preliminary Report.* (2020). Available online at: https://www.medrxiv.org/content/10.1101/2020.06.22.20137273v1.full.pdf (accessed June 23, 2020).
110. World Health Organization. *WHO Welcomes Preliminary Results About Dexamethasone Use in Treating Critically Ill COVID-19 Patients.* (2020). Available online at: https://www.who.int/news-room/detail/16-06-2020-who-welcomes-preliminary-results-about-dexamethasone-use-in-treating-critically-ill-covid-19-patients (accessed June 23, 2020).
111. Duan K, Liu B, Li C, Zhang H, Yu T, Qu J, et al. Effectiveness of convalescent plasma therapy in severe COVID-19 patients. *Proc Natl Acad Sci USA*. (2020) 117:9490–6. doi: 10.1073/pnas.2004168117
112. Shen C, Wang Z, Zhao F, Yang Y, Li J, Yuan J, et al. Treatment of 5 critically ill patients with COVID-19 with convalescent plasma. *JAMA*. (2020) 323:1582–89. doi: 10.1001/jama.2020.4783
113. U. S., and Food Drug Administration. *Investigational COVID-19 convalescent plasma - emergency INDs.* (2020). Available online at: https://www.fda.gov/vaccines-blood-biologics/investigational-new-drug-ind-or-device-exemption-ide-process-cber/recommendations-investigational-covid-19-convalescent-plasma (accessed April 13 2020).
114. Thachil J, Tang N, Gando S, Falanga A, Cattaneo M, Levi M, et al. ISTH interim guidance on recognition and management of coagulopathy in COVID-19. *J Thromb Haemost.* (2020) 18:1023–26. doi: 10.1111/jth.14810
115. Boretti A, Banik BK. Intravenous vitamin C for reduction of cytokines storn in acute respiratory distress syndrome. *Pharma Nutr.* (2020) 12:100190. doi: 10.1016/j.phanu.2020.100190
116. Fowler AA, Truwit JD, Hite RD, Morris PE, DeWilde C, Priday, A. Effect of vitamin C. *JAMA*. (2019) 322:1261–70. doi: 10.1001/jama.2019.11825
117. ClinicalTrials.gov. *Vitamin C Infusion for the Treatment of Severe 2019-nCoV Infected Pneumonia*. (2020). Available online at: https://clinicaltrials.gov/ct2/show/NCT04264533 (accessed June 23, 2020).

118. Te Velthuis AJ, Van Den Worm SH, Sims AC, Baric RS, Snijder EJ, Van Hemert MJ. Zn(2+) inhibits coronavirus and arterivirus RNA polymerase activity *in vitro* and zinc ionophores block the replication of these viruses in cell culture. *PLoS Pathog.* (2010) 6:e1001176. doi: 10.1371/journal.ppat.1001176
119. ClinicalTrials.gov. *A Study of Hydroxychloroquine, Vitamin C, Vitamin D, and Zinc for the Prevention of COVID-19 Infection (HELPCOVID-19).* (2020). Available online at: https://clinicaltrials.gov/ct2/show/NCT04335084 (accessed June 23, 2020).
120. Fidan C, Aydogdu A. As a potential treatment of COVID-19: montelukast. *Med Hypotheses.* (2020) 142:109828. doi: 10.1016/j.mehy.2020.109828
121. ClinicalTrials.gov. *The COVID-19 Symptom Motelukast Trial (COSMO).* (2020). Available online at: https://clinicaltrials.gov/ct2/show/NCT04389411 (accessed June 23, 2020).
122. Le TT, Andreadakis Z, Kumar A, Román RG, Tollefsen S, Saville M, et al. The COVID-19 vaccine development landscape. *Nat Rev Drug Disc.* (2020) 19:305–6. doi: 10.1038/d41573-020-00073-5
123. ClinicalTrials.gov. *Safety and Immunogenicity Study of 2019-nCoV Vaccine (mRNA-1273) for Prophylaxis SARS CoV-2 Infection (COVID-19).* (2020). Available online at: https://clinicaltrials.gov/ct2/show/NCT04283461 (accessed April 16, 2020).
124. Patel A, Jernigan DB, Nco VCDCRT. Initial public health response and interim clinical guidance for the 2019 novel coronavirus outbreak - United States, December 31, 2019-February 4, 2020. *MMWR Morb Mortal Wkly Rep.* (2020) 69:140–6. doi: 10.15585/mmwr.mm6905e1
125. Sohrabi C, Alsafi Z, O'neill N, Khan M, Kerwan A, Al-Jabir A, et al. World Health Organization declares global emergency: a review of the 2019 novel coronavirus (COVID-19). *Int J Surg.* (2020) 76:71–6. doi: 10.1016/j.ijsu.2020.02.034
126. Phelan AL, Katz R, Gostin LO. The novel coronavirus originating in Wuhan, China: challenges for global health governance. *JAMA.* (2020) 323:709–10. doi: 10.1001/jama.2020.1097
127. Gostin LO, Hodge JG Jr. US emergency legal responses to novel coronavirus: balancing public health and civil liberties. *JAMA.* (2020) 323:1131–2. doi: 10.1001/jama.2020.2025
128. CDC. *Implementation of Mitigation Strategies for Communities With Local COVID-19 Transmission.* (2020). Available online at: https://www.cdc.gov/coronavirus/2019-ncov/downloads/community-mitigation-strategy.pdf (accessed April 18, 2020).
129. Friston KJ, Parr T, Zeidman P, Razi A, Flandin G, Daunizeau J, et al. *Second Waves, Social Distancing, and the Spread of COVID-19 Across America.* (2020). Available at: https://wellcomeopenresearch.org/articles/5-103/v1 (accessed June 27, 2020).
130. Aleta A, Martin-Corral D, Piontti AP, Ajelli M, Litvinova M, Chinazzi M, et al. *Modeling the Impact of Social Distancing, Testing, Contact Tracing and Household Quarantine on Second-Wave Scenarios of the COVID-19 Pandemic.* (2020). Available at: https://www.medrxiv.org/content/10.1101/2020.05.06.20092841v1 (accessed June 27, 2020).
131. Hick JL, Biddinger PD. Novel coronavirus and old lessons - preparing the health system for the pandemic. *N Engl J Med.* (2020) 382:e55. doi: 10.1056/NEJMp2005118

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 31 July 2020
doi: 10.3389/fmicb.2020.01858

CoronaVR: A Computational Resource and Analysis of Epitopes and Therapeutics for Severe Acute Respiratory Syndrome Coronavirus-2

*Amit Kumar Gupta[1†], Md. Shoaib Khan[1†], Shubham Choudhury[1†], Adhip Mukhopadhyay[1,2†], Sakshi[1,2†], Amber Rastogi[1,2†], Anamika Thakur[1,2], Pallawi Kumari[1], Manmeet Kaur[1], Shalu[1], Chanchal Saini[1], Vandna Sapehia[1], Barkha[1], Pradeep Kumar Patel[1], Kailash T. Bhamare[1] and Manoj Kumar[1,2]**

[1] Virology Unit and Bioinformatics Centre, Institute of Microbial Technology, Council of Scientific and Industrial Research (CSIR), Sector 39-A, Chandigarh, India, [2] Academy of Scientific and Innovative Research (AcSIR), Ghaziabad, India

OPEN ACCESS

Edited by:
Lars Kaderali,
Universitätsmedizin Greifswald, Germany

Reviewed by:
Silvia Beatriz Boscardin,
University of São Paulo, Brazil
Vikas Kaushik,
Lovely Professional University, India

***Correspondence:**
Manoj Kumar
manojk@imtech.res.in

† These authors have contributed equally to this work

Specialty section:
This article was submitted to Virology, a section of the journal Frontiers in Microbiology

Received: 10 April 2020
Accepted: 15 July 2020
Published: 31 July 2020

Citation:
Gupta AK, Khan MS, Choudhury S, Mukhopadhyay A, Sakshi, Rastogi A, Thakur A, Kumari P, Kaur M, Shalu, Saini C, Sapehia V, Barkha, Patel PK, Bhamare KT and Kumar M (2020) CoronaVR: A Computational Resource and Analysis of Epitopes and Therapeutics for Severe Acute Respiratory Syndrome Coronavirus-2. Front. Microbiol. 11:1858. doi: 10.3389/fmicb.2020.01858

In December 2019, the Chinese city of Wuhan was the center of origin of a pneumonia-like disease outbreak with an unknown causative pathogen. The CDC, China, managed to track the source of infection to a novel coronavirus (2019-nCoV; SARS-CoV-2) that shares approximately 79.6% of its genome with SARS-CoV. The World Health Organization (WHO) initially declared COVID-19 as a Public Health Emergency of International Concern (PHEIC) and later characterized it as a global pandemic on March 11, 2020. Due to the novel nature of this virus, there is an urgent need for vaccines and therapeutics to control the spread of SARS-CoV-2 and its associated disease, COVID-19. Global efforts are underway to circumvent its further spread and treat COVID-19 patients through experimental vaccine formulations and therapeutic interventions, respectively. In the absence of any effective therapeutics, we have devised h bioinformatics-based approaches to accelerate global efforts in the fight against SARS-CoV-2 and to assist researchers in the initial phase of vaccine and therapeutics development. In this study, we have performed comprehensive meta-analyses and developed an integrative resource, "CoronaVR" (http://bioinfo.imtech.res.in/manojk/coronavr/). Predominantly, we identified potential epitope-based vaccine candidates, siRNA-based therapeutic regimens, and diagnostic primers. The resource is categorized into the main sections "Genomes," "Epitopes," "Therapeutics," and Primers." The genome section harbors different components, viz, genomes, a genome browser, phylogenetic analysis, codon usage, glycosylation sites, and structural analysis. Under the umbrella of epitopes, sub-divisions, namely cross-protective epitopes, B-cell (linear/discontinuous), T-cell ($CD4^+$/$CD8^+$), CTL, and MHC binders, are presented. The therapeutics section has different sub-sections like siRNA, miRNAs, and sgRNAs. Further, experimentally confirmed and designed diagnostic primers are earmarked in the primers section. Our study provided a set of shortlisted B-cell and T-cell ($CD4^+$

and $CD8^+$) epitopes that can be experimentally tested for their incorporation in vaccine formulations. The list of selected primers can be used in testing kits to identify SARS-CoV-2, while the recommended siRNAs, sgRNAs, and miRNAs can be used in therapeutic regimens. We foresee that this resource will help in advancing the research against coronaviruses.

Keywords: SARS-CoV-2, 2019-nCoV, COVID-19, epitopes, therapeutics, primers

INTRODUCTION

The world is currently undergoing and living with the great threat of pathogenic severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), which has newly emerged from Wuhan, Hubei province, China (Du Toit, 2020; Hui et al., 2020; Wang C. et al., 2020). Apart from this, in recent years, we have also witnessed sporadic outbreaks and epidemics of various lethal viruses, i.e., Ebola, Zika, Nipah, etc (Gupta et al., 2016, 2020). The current pandemic of SARS-CoV-2 (also named as 2019-nCoV) is now reported to spread over 199 countries and to be responsible for excessive economic loss worldwide (Zhang and Liu, 2020). The World Health Organization (WHO) declared it a public health emergency with a global alert (Ryu and Chun, 2020; Wang C. et al., 2020). Overall, more than 10 million cases and over 0.5 million deaths had been reported worldwide by the end of June 2020[1]. Earlier in different years, CoVs have emerged periodically in various regions worldwide with different death rates (Ksiazek et al., 2003; Bogoch et al., 2020; Guarner, 2020). During the epidemic in 2002–2003, severe acute respiratory syndrome coronavirus (SARS-CoV) led to reported deaths and infected cases of 916 and 8422, respectively. Likewise, another outbreak of Middle East respiratory syndrome coronavirus (MERS-CoV) was reported in 2012, with 543 deaths out of 1401 total cases, giving it a mortality rate of around ∼39% (de Wit et al., 2016).

Coronaviruses (CoVs) are positive-sense single-stranded enveloped RNA viruses belonging to the *Coronaviridae* family (The, 2020). CoVs are the largest known RNA virus genomes, being 27 to 32 kb in length. CoV genomes contains 10–12 open reading frames (ORFs) that encode for the four structural proteins, i.e., surface glycoprotein (or spike) (S), envelope (E), membrane glycoprotein (M), and nucleocapsid (N), 16 non-structural proteins (NSP1–NSP16) (orf1ab polyprotein), other accessory proteins like ORF3a, ORF6, ORF7a, ORF7b, ORF8, and ORF10. These are only RNA viruses, which encode proofreading machinery, i.e., exonuclease and other replicase proteins, for the regulation of fidelity (The, 2020).

Coronaviruses are genotypically divided into four genera, viz., alpha, beta, gamma, and delta coronaviruses. Among these, beta coronaviruses are further classified into four subgroups, i.e., A, B, C, and D (Lu et al., 2020). Previously, six CoVs, two from the alpha group (HCoV-229E and HCoV-NL63) and four belonging to the beta group [HCoV-HKU1 (subgroup-A), HCoV-OC43 (A), SARS-CoV (subgroup-B), MERS-CoV (subgroup-C)], were known to infect humans. SARS-CoV-2 becomes the seventh coronavirus member to infect humans (Cheng and Shan, 2020; Zhu et al., 2020). CoVs are highly pathogenic agents known to cause mainly fatal respiratory ailments (like pneumonia) and to infect various species like humans, bats, pigs, etc (Huang et al., 2020; Lu et al., 2020; Wang C. et al., 2020). Common symptoms are fever, cough, fatigue, breath shortness, muscle ache, headache, diarrhea, etc (Chen et al., 2020; Del Rio and Malani, 2020; Huang et al., 2020; Wu et al., 2020).

Different strategies have been trialed and applied to combat these viruses (Dennis Lo and Chiu, 2020; Maxmen, 2020; Watts et al., 2020; Zhang J. et al., 2020). Primarily, four proteins, which include two proteases, i.e., coronavirus main proteinase (3CLpro) and papain-like protease (PLpro), which are responsible for the proteolysis process, a replicase RNA-dependent RNA polymerase (RdRp) responsible for the replication of RNA genome, and surface glycoprotein (spike), which mediates viral entry and fusion to host cells, are essential for the CoVs, making them preferred targets for therapeutics (Du et al., 2017; Cheng and Shan, 2020; Goo et al., 2020; Morse et al., 2020; Zhang J. et al., 2020). Researchers have mainly explored the ability of existing FDA-approved drugs to control SARS-CoV-2 (Zumla et al., 2016; Lu, 2020). For example, Wang et al., has shown that Remdesivir (GS-5734), a nucleotide prodrug, and Chloroquine effectively inhibit 2019-nCoV *in vitro* (Colson et al., 2020; Wang M. et al., 2020). Remdesivir is known to exhibit broad antiviral activity and has also previously been shown to have effective inhibition efficiency against MERS-CoV, SARS-CoV, Ebola, and Nipah (de Wit et al., 2020; Lu, 2020; Sheahan et al., 2020). Further, various antiviral agents are also in separate clinical trials targeting different SARS-CoV-2 genomic regions/proteins (Maxmen, 2020).

Furthermore, different studies have also reported potential inhibitors to combat CoVs (Momattin et al., 2019; Shen et al., 2019; Totura and Bavari, 2019; Xia et al., 2019). Various studies have also shown the use of different vaccine candidates primarily based on the spike (S), nucleocapsid (N), and envelope (E) proteins (Schoeman and Fielding, 2019; Yong et al., 2019; Zumla et al., 2019; Goo et al., 2020; Tian et al., 2020).

Additionally, various groups have also advocated the use of immune-informatics and computational approaches to target the different proteins of CoVs (SARS as well as MERS). For example, Qamar et al., provide B- and T-cell epitopes against the MERS-CoV spike (S) protein (Tahir Ul Qamar et al., 2019). Srivastava et al., used the *in silico* method to design a multi-epitope vaccine (MEV) against MERS-CoV and SARS-CoV (Srivastava et al., 2018, 2019). Shi et al. (2015) have screened epitope-based vaccine targets against MERS-CoV. Another study provides N protein-based B and CTL epitopes against MERS-CoV

[1]https://www.who.int/docs/default-source/coronaviruse/20200630-covid-19-sitrep-162.pdf?sfvrsn=e00a5466_2

(Hori et al., 1989). Recently, a report identified T-cell and B-cell epitopes in the surface glycoprotein of 2019-nCoV (Baruah and Bose, 2020). However, there is no approved drug and licensed vaccine available to combat the virus. Therefore, effective control strategies are urgently required to combat this deadly pathogen (Kickbusch and Leung, 2020; Lu, 2020; Zhang and Liu, 2020). To support the global efforts to fight this virus, we have performed an *in silico* analyses and developed a resource of vaccine candidates and therapeutics to assist the global scientific community.

MATERIALS AND METHODS

Data Collection and Curation

The aim of the current work and analysis is to target all of the Human infecting coronaviruses, with a prime focus on SARS-CoV-2. Complete genome sequences of the CoVs having Humans as hosts were retrieved from the NCBI. An advanced search interface is also deployed on the server to serve the users' requirements. Along with this, we have also implemented a genome browser for interactive graphical visualization utilizing JBrowse (Buels et al., 2016). Further, as the world is currently suffering from the outbreak of SARS-CoV-2, we have primarily concentrated on the alternative therapeutic options and vaccine candidates. For this, we mainly utilized the protein and gene sequences of the reference SARS-CoV-2 (NC_045512.2). We have also explored the cross targeting and conservancy of different putative regimens against the other six reference CoVs, namely, SARS-CoV (NC_004718.3), MERS-CoV (NC_019843.3), HCoVs NL63 (NC_005831.2), HCoVs 229E (NC_002645.1), HCoVs OC43 (NC_006213.1), and HCoVs HKU1 (NC_006577.2).

Vaccine Epitopes

The sequences of a large polyprotein (ORF1ab), four structural proteins [Envelope (E), Spike (S), Nucleocapsid (N), and Membrane (M)], and accessory proteins (ORF3a, ORF6, ORF7a, ORF7b, ORF8, and ORF10) of annotated SARS-CoV-2 (NC_045512.2) were retrieved and utilized for the analysis. These sequences were used to predict putative T-cell epitopes (MHC-I and MHC-II binders, Cytotoxic T-lymphocytes (CTL), and Immunogenic $CD8^+$ and $CD4^+$ epitopes) and B-cell epitopes (linear and conformational) that can be used for designing vaccines against CoVs. An overview of the epitope analysis pipeline is depicted in **Figure 1**.

T-Cell Epitope Prediction

We predicted MHC-I and MHC-II binders, CTL epitopes, immunogenic $CD8^+$ and $CD4^+$ T-cell epitopes, and IFN-γ-inducing peptides (restricted by MHC-II) from all protein sequences of SARS-CoV-2.

For MHC-I and MHC-II binding prediction, we used the corresponding tools available at the Immune Epitope Database (IEDB) Epitope analysis tool page[2] (Peters and Sette, 2005; Nielsen et al., 2007). For this, the "IEDB recommended" approach was utilized. This approach adopts a consensus method comprising ANN, SMM, and CombLib (if the predictor is available for a particular HLA; otherwise, it uses NetMHCpan EL) for MHC-I and NN-align, SMM-align, CombLib, and Sturniolo for MHC-II (Kim et al., 2012). Shortlisting of predicted binders can be done based on percentile ranks and predicted affinities, where peptides with low percentile rank and low-affinity value (IC50 < 50 nM) are considered good binders (Kim et al., 2012).

The prediction of $CD8^+$ (CTL) T-cell epitopes was performed using NetCTLpan *v* 1.1 Server[3] for 12 HLA supertypes (A1, A2, A3, A24, A26, B7, B8, B27, B39, B44, B58, and B62) (Stranzl et al., 2010). While predicting CTL epitopes, it takes into account various sequence-processing steps such as cleavage by proteasomes, TAP binding, and MHC-I binding (Stranzl et al., 2010). MHC-I-restricted immunogenic peptides were identified using the "IEDB Class I Immunogenicity tool"[4] with the default settings (Calis et al., 2013). This is based on amino acid properties and their respective positions within the sequence and gives an output in the form of scores, where a higher score indicates a greater probability of eliciting an immune response (Calis et al., 2013).

The immunogenicity of MHC-II restricted peptides was predicted using the "CD4 T cell immunogenicity prediction tool" available at the IEDB[5]. The prediction was performed with the "IEDB recommended" method, which uses a combination of MHC-binding to seven alleles and the immunogenicity method (Dhanda et al., 2018). The output is in the form of a table containing a description of the input sequences along with the combined score, immunogenicity score, 9-mer peptide core, median percentile rank, and score for each of the seven alleles.

Furthermore, Interferon-gamma (IFN-γ) is secreted by T-helper cells and is of central help in clearing the viruses from the host (Chesler and Reiss, 2002). IFN-γ-inducing peptides were predicted among positive MHC-II binders (15-mer) using the IFNepitope web server[6] (Dhanda et al., 2013). The default settings ("motif and SVM hybrid" and the "IFN-gamma vs. Non-IFN-gamma" model) were used to predict IFN-γ-inducing peptides based on score, where the higher the score, the higher the chance of inducing IFN-γ (Dhanda et al., 2013).

B-Cell Epitope Prediction

The identification of linear (continuous) B-cell epitopes is an important step in designing a vaccine against a microorganism. Linear B-cell epitope prediction was accomplished using the "BepiPred Linear Epitope Prediction 2.0" method available at the B-cell epitope prediction tool of the IEDB[7]. The tool is based on the random forest algorithm and was trained on amino acids of epitopes and non-epitopes identified from antigen-antibody crystal structures (Jespersen et al., 2017). Amino acid residues with scores greater than the default threshold value of 0.5 are envisaged as being part of an epitope (Jespersen et al., 2017).

[2]http://tools.iedb.org/main/tcell/

[3]http://www.cbs.dtu.dk/services/NetCTLpan/

[4]http://tools.iedb.org/immunogenicity/

[5]http://tools.iedb.org/CD4episcore/

[6]http://crdd.osdd.net/raghava/ifnepitope/

[7]http://tools.iedb.org/main/bcell/

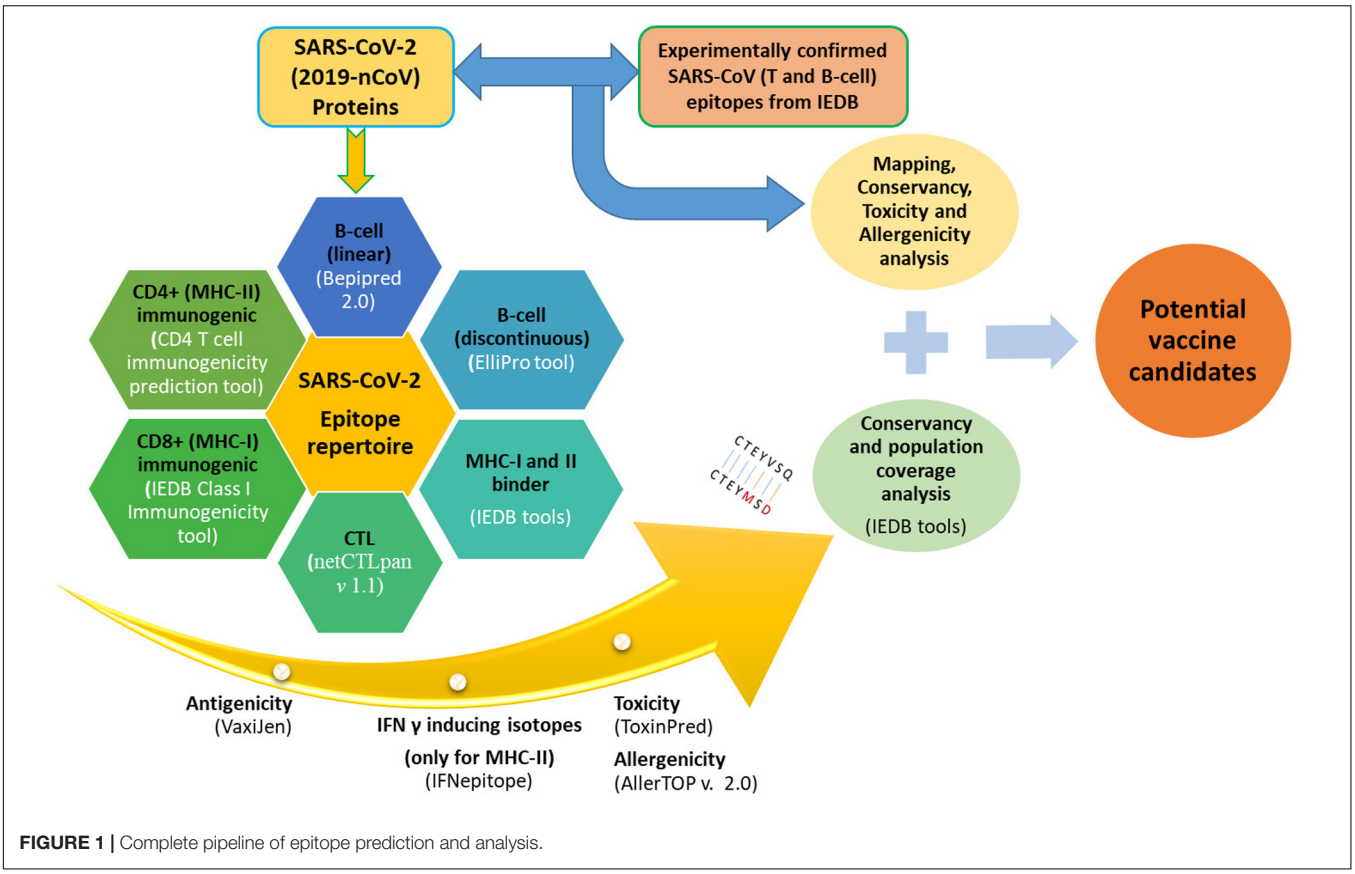


FIGURE 1 | Complete pipeline of epitope prediction and analysis.

The conformational B-cell epitopes are discontinuous or scattered amino acid sequences that make up an antigen and interact with B-cell receptors (BCR) (Sanchez-Trincado et al., 2017). Prediction of these discontinuous B-cell epitopes was performed using the ElliPro tool available at the IEDB[8] (Ponomarenko et al., 2008). It predicts discontinuous B-cell epitopes based on the 3D structure of protein antigen depending on selected parameters, with the defaults being 0.5 and 6 Angstrom (Å) for minimum score and maximum distance, respectively (Ponomarenko et al., 2008). The output result is in the form of a table displaying "amino acid residues," "Number of residues," "Score," and a link to "3D structure" (Ponomarenko et al., 2008).

Feature Profiling of Selected B- and T-Cell Epitopes

The shortlisted predicted epitopes (B-cell and T-cell) were analyzed for important features such as antigenicity, toxicity, and allergenicity. The probable peptide-based vaccine epitopes must be antigenic, non-toxic, and non-allergenic.

Antigenicity Prediction

Antigenicity prediction of the selected epitopes was performed to find the antigenic peptides. To accomplish this, we used the Vaxijen v2.0 server[9] to predict the antigenicity of these predicted MHC-I and MHC-II binders, CTL epitopes, immunogenic $CD8^+$ and $CD4^+$ T-cell epitopes, and linear B-cell epitopes. Vaxijen v2.0 was used with a default cut-off of 0.4, indicative of viral antigens, to assess the antigenicity of these peptides (Doytchinova and Flower, 2007).

Toxicity and Allergenicity Prediction

The toxicity of antigenic B-cell and T-cell epitopes with a Vaxijen score above 0.4 was predicted using the ToxinPred web server[10] (Gupta et al., 2013). It is based on a quantitative matrix and Support Vector Machine (SVM) utilizing various peptide properties (Gupta et al., 2013). We used the SVM (Swiss-Prot)-based method while keeping all other criteria as default. Epitopes with the prediction result "Non-toxin" were used for further analysis. Likewise, putative vaccine candidates must be checked for allergenicity to prevent allergic responses in the host that may be caused by vaccination (McKeever et al., 2004). We used AllerTOP v. 2.0[11] to predict the allergenicity of the epitopes being forecasted as "Non-toxic" by ToxinPred. This was developed based on using the k-nearest neighbors (kNN) method to discriminate allergens from non-allergens (Dimitrov et al., 2014).

[8]http://tools.iedb.org/ellipro/

[9]http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html

[10]http://www.imtech.res.in/raghava/toxinpred/

[11]http://www.ddg-pharmfac.net/AllerTOP/

Epitope Conservancy Analysis

The conservancy of the predicted epitopes was further analyzed using the epitope conservancy tool available at the IEDB[12] (Bui et al., 2007). Conservancy is an indication of the percentage identity of the selected epitopes with the proteins of other similar organisms (here, other coronaviruses). We tested the conservancy of predicted epitopes with the other six coronavirus strains that are responsible for causing respiratory illnesses in humans, comprising two alpha coronaviruses (NL63 and 229E) and four beta coronaviruses (SARS, MERS, OC43, and HKU1).

Population Coverage Analysis

The numerous polymorphic HLAs present in different populations have varied frequencies, and the epitopes restricted by such HLAs would have biased population coverage (Sidney et al., 2010). Hence, during a vaccine design, population coverage must be accounted for to avoid a decrease in the applicability of a vaccine candidate in some populations (Bui et al., 2006). Therefore, it is vital to calculate the frequency of individuals that are anticipated to respond to a given epitope set based on HLA typing (Bui et al., 2006).

We further analyzed the population coverage of the predicted $CD8^+$ (MHC-I), $CD4^+$ (MHC-II), and CTL epitopes and their respective HLA alleles using the IEDB population coverage tool[13] (Bui et al., 2006). This reflects the percentage of individuals in a population likely to respond to at least one T-cell epitope from the collection (Bui et al., 2006). The "HLA–epitope pairs" set (epitopes with their restricted HLA alleles) was utilized to compute the projected population coverage (PPC) using query-"area_country_ethnicity" and selecting each of the 16 areas to provide broad global coverage, including China.

Coronavirus Derived T- and B-Cell Epitopes

The T-cell (MHC class I and class II) and B-cell epitopes of all coronaviruses around the world were searched in the IEDB by querying "Coronavirus" (taxonomy ID: 11118). The search was restricted to "Positive Assays Only" for both "T-cell Assays" and "B-cell Assays" for "Any Host," "Any MHC restriction," and "Any Disease."

SARS-CoV Derived T- and B-Cell Epitopes

The T-cell (MHC class I and class II) and B-cell epitopes of SARS-CoV were explored in the IEDB by querying "Severe acute respiratory syndrome-related coronavirus (taxonomy ID: 694009). We restricted our search to "Linear Epitope" and "Positive Assays Only" to include linear epitopes with at least one positive assay for T cell and B cell, respectively, while keeping all other parameters as default.

[12]http://tools.iedb.org/conservancy/

[13]http://tools.iedb.org/population/

RNAi-Based Therapeutics

Potential Small Interfering RNAs (siRNAs)

We used the VIRsiRNApred (Qureshi et al., 2013) and desiRm (Ahmed and Raghava, 2011) programs for the prediction of siRNAs against SARS-CoV-2. VIRsiRNApred is a virus-specific method, and we used model-2, constructed by employing different features like the hybrid nucleotide frequencies, binary pattern, and thermodynamic properties of 1725 viral siRNAs. Further, only highly efficacious siRNAs (inhibition more than or equal to 60%) were considered. Additionally, potential siRNAs (predicted efficacy score greater or equal to 1) were also identified using the desiRm tool. Moreover, the off-targets of the siRNAs were also predicted. Additionally, the immunomodulatory impact was also deduced by the imRNA tool, which explores the immunomodulatory and non-immunomodulatory potential of siRNAs (Nagpal et al., 2017).

Putative MicroRNAs (miRNAs)

Similarly, we have also identified miRNAs for SARS-CoV-2 using a two-step method. In the first step, the VMir algorithm was utilized to predict the precursor miRNA (pre-miRNA) hairpins using the default parameters (Sullivan and Grundhoff, 2007), while in the second step, mature miRNAs were identified using the Mature Bayes tool (Gkirtzou et al., 2010).

Single Guide RNAs (sgRNAs)

For the identification of all of the possible single guide RNAs (sgRNAs), we used the ge-CRISPR tool/pipeline (Kaur et al., 2016). Prediction of sgRNAs was performed based on the Protospacer Adjacent motif (PAM) for the SARS-CoV-2 genome. The underlying algorithm scans all the "NGG" motifs in the genome for both the forward and reverse strands and picks up putative sgRNAs 20 nucleotides upstream of the motifs found thereby. In the geCRISPR tool pipeline 2, ge-CRISPRr was selected, which employs a regression-based algorithm to predict sgRNA efficiency (0–100%).

Coronavirus (CoV) Primers

To obtain an exhaustive list of primers, two separate approaches were employed in the study. First, we searched for the experimental primers previously used for the detection of coronaviruses (CoVs). For this, a literature search was performed in PubMed using the different keywords "coronavirus" and "primers*." Overall, 185 papers were obtained (on 12/02/2020) and were further examined to collect the oligonucleotide primer information. Meta-information was collected for each primer pair, mainly primer name, sequence, orientation, start-end, genome name, gene name, strain, accession number, etc.

Furthermore, in the second approach, we designed primer pairs for SARS-CoV-2 based on different parameters using the PrimerDesign-M tool (Yoon and Leitner, 2015). We used the multiple fragment option with Flex design for fragment overlap. Further, the start and end of the target region were specified for the region of interest. Additionally, primer length range (20–25), detection limit (5%), complexity limit (2%, one degenerate position), window size (10-mer), and dimer ratio (0.9) were used.

A 5°C difference between the melting temperatures (Tm) of the forward and reverse primer in pairs was set.

Glycosylation in CoVs

We also performed prediction and analysis of glycosylation sites (C, N, and O) for all of the proteins of SARS-CoV-2. Additionally, the other six CoVs, i.e., SARS, MERS, 229E, OC43, NL63, and HKU1, were also investigated for the identification of glycosylation sites. We used NetCGlyc*v*1.0 (Julenius, 2007), NetNGlyc*v*1.0 (Blom et al., 2004), and NetOGlyc *v*.4.0 (Steentoft et al., 2013) for C-linked, N-linked, and O-linked glycosylation, respectively. Additionally, we also compared the glycosylation sites in these seven CoVs to elucidate the conservation between them.

Phylogenetics

For the phylogenetic analysis, 48 representative coronavirus genomes and their corresponding proteomes (latest as of 17/02/2020) were selected, and their evolutionary relationship was identified using MEGA 10.1.7 (Kumar et al., 2018). Genome sequence alignment was performed using the MUSCLE (Edgar, 2004) algorithm integrated within the MEGA program. For both the genomes and the proteomes, the phylogenetic tree was constructed based on the maximum likelihood (ML) method. In the case of genomes, the ML tree was constructed following the general time-reversible (GTR) model using a discrete Gamma distribution (+G). Similarly, for proteomes, the LG (Le and Gascuel, 2008) model using discrete Gamma distribution (+G) was used for building the ML tree. The robustness of the tree topology was calculated using the bootstrap method (Felsenstein, 1985) with 1000 bootstrap replications for the genome-based tree, while the corresponding proteome tree was built using 100 bootstrap replicates.

Codon Usage and Nucleotide Composition

Complete nucleotide sequences of all coding regions of SARS-CoV-2 were retrieved from NCBI (NC_045512.2). To gain insight into the codon usage, different parameters such as the number of amino acids, number of codons, relative synonymous codon usage (RSCU), rare codons, and codon context were calculated using Anaconda software (Moura et al., 2005). The nucleotide composition (in percentages) of A, U, G, C, A + U, G + C, G + A, G + T, A + T, A + C, C + T, GC1, GC2, and GC3 of all coding regions was calculated using the online program CAIcal[14] (Puigbo et al., 2008). Additionally, the estimation of codon adaptation of the SARS-CoV-2 in the host, the effective number of codons (ENc), and the Codon Adaptation Index (CAI) value were calculated using CAIcal software. In the analysis, the synonymous codon usage pattern of the viral host *(Homo sapiens)* was taken as the reference, and the CAI values of the coding regions of SARS-CoV-2 were calculated after comparison with the reference. The codon usage pattern of *Homo sapiens* was retrieved from the Codon Usage Database[15].

[14]http://genomes.urv.es/CAIcal/

[15]https://www.kazusa.or.jp/codon/

Protein Structure Prediction, Comparison, and Analysis

In order to elucidate important aspects and structural conservation of SARS-CoV-2 proteins, *in silico* structure prediction and analysis was performed for six proteins of CoVs, namely, the four structural proteins, S, E, N, and M, and two non-structural proteins, RNA-dependent RNA polymerase (RdRp) and Helicase. The structures of the above-mentioned proteins from seven different CoVs were modeled using SWISS-MODEL (Waterhouse et al., 2018). Further, 3D structural comparison and analysis were also performed and represented using PyMOLv1.7.4[16]. All of the predicted structures of proteins for these seven CoVs, including all SARS-CoV-2 proteins, are also provided on our web resource with the visualization and download facility.

CoronaVR Resource Development

"CoronaVR" was built and hosted in the Linux environment on an Apache HTTP server (v2.2.17) utilizing the LAMP (Linux, Apache HTTP Server, MySQL, and PHP) open-source platform. The backend is mainly supported by MySQL for effective data management. The web-interface was created the employing PHP, HTML, CSS, and JavaScript. In-house scripts were also developed to process and perform data processing. Further, a Corona genome browser was also included.

RESULTS AND DISCUSSION

We have developed an integrative resource equipped with a compendium of putative anti-CoV solutions and genomic knowledge to assist the scientific community in dealing with the deadly public health threat of COVID-19. For this, using a systematic and dedicated approach, we developed "CoronaVR." The resource is well-organized into different sections for interactive navigation. It is broadly categorized into the separate divisions, viz., epitopes, therapeutics, primers, and genomes. It also comprises tools for analysis and visualization. A complete overview of the CoronaVR resource is illustrated in **Figure 2**.

CoronaVR Genomes and Browser

We have compiled 365 complete genome sequences of human infective CoVs with sizes ranging between 27 and 32 kb. A catalog of CoVs is also provided in the resource in the genomes section. A categorywise advance search facility using different criteria, viz., geographic area (e.g., Asia), country (e.g., China), Year (2003, 2019, etc.), Length range, etc., is also implemented for sequence data retrieval. Detailed meta-information, like genome accession number, virus name, strain/isolate, length, geographical area, country of origin, etc., is provided. To navigate through the seven reference human-infecting CoVs, we have also developed a graphical genome browser backed by JBrowse. Different color codes depict distinct genome features with semantic navigation, a ruler, and zooming.

[16]https://pymol.org/2/

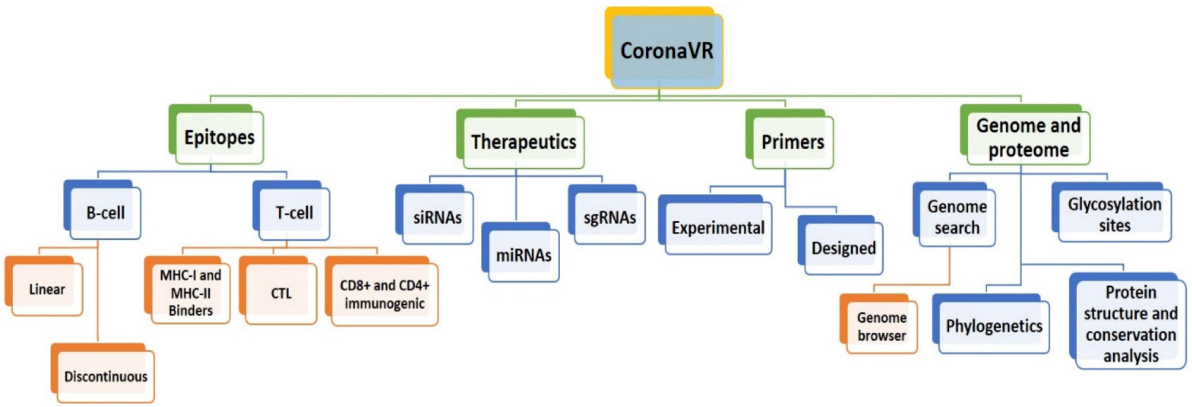


FIGURE 2 | Architecture of the CoronaVR compendium.

Putative Vaccine Epitopes

We used the IEDB MHC-I binding prediction tool to predict MHC-I binders from protein sequences of SARS-CoV-2. The consensus method was used for binding prediction, and peptides with IC50 less than 50 nM were selected as strong binders. These predicted binders of each protein sequence are subjected to antigenicity, toxicity, and allergenicity prediction. Out of the total 424 non-allergenic, non-toxic, and antigenic MHC-I binders, 168 peptides were found to be 100% conserved within the SARS-CoV protein sequences (**Supplementary Table S1**). The number of peptide sequences that remained after each prediction step is shown in **Supplementary Table S2**.

Likewise, we used NetCTLpan *v*1.1 to predict $CD8^+$ T-cell epitopes from protein sequences. Prediction was made on 12 HLA supertypes (A1, A2, A3, A24, A26, B7, B8, B27, B39, B44, B58, and B62) with the remaining parameters as default. The peptides with a% Rank less than 1% (<E) were selected as per the default selection criteria of the web server. Overall, 1499 CTL epitopes were predicted from 11 proteins of SARS-CoV-2. Out of 1499 predicted CTL epitopes, 765 were found to be antigenic. Further analysis of these 765 peptides showed that 754 were non-toxic and 273 were non-allergenic (**Supplementary Tables S3, S4**). These 273 non-allergenic CTL epitopes were analyzed for sequence conservancy and population coverage. Of the 273, 169 epitopes were found to be 100% conserved with SARS-CoV sequences, while the others were conserved to variable degrees (**Supplementary Table S3**). Potential CTL epitopes pertaining to the four structural proteins (E, S, M, and N) conserved in both SARS-CoV-2 and SARS-CoV are provided in **Table 1**.

Furthermore, immunogenic peptides restricted to MHC-I were identified using the "IEDB Class I Immunogenicity tool" with default parameters. We found 236 immunogenic epitopes in total, with envelope (E) and ORF8 having no predicted immunogenic MHC-I epitopes. Out of these 236 epitopes, only 33 were found to be antigenic according to the Vaxijen score and were selected for toxicity and allergenicity prediction. These 33 peptides were found to be non-toxic, while 21 were non-allergenic (**Supplementary Table S5**). The numbers of selected epitopes from each protein used for prediction at each step are shown in **Supplementary Table S6**. Finally, these 21 immunogenic and non-allergenic epitopes from different proteins were selected for conservancy analysis and population coverage (**Table 2**). The conservancy analysis showed that only two immunogenic CD8 + T-cell epitopes (present in ORF7b) were 100% conserved with the SARS-CoV sequences, while five were 90% conserved (**Supplementary Table S5**).

Similarly, MHC-II binders from SARS-CoV-2 protein sequences were predicted using the IEDB MHC-II binding prediction method. As per IEDB recommendation, we used

TABLE 1 | Potential CTL epitopes conserved in SARS-CoV-2 and SARS-CoV.

Protein	*Peptides*	*Start*	*Stop*
E	SVLLFLAFV	16	24
E	LLFLAFVVF	18	26
E	FLAFVVFLL	20	28
E	FLLVTLAIL	26	34
E	YVYSRVKNL	57	65
M	LWPVTLACF	57	65
M	FVLAAVYRI	65	73
M	SELVIGAVI	136	144
M	ATSRTLSYY	173	181
M	TSRTLSYYK	174	182
N	LSPRWYFYY	105	113
N	SPRWYFYYL	106	114
N	KTFPPTEPK	366	374
S	VRFPNITNL	331	339
S	YQPYRVVVL	512	520
S	PYRVVVLSF	514	522
S	LLFNKVTLA	832	841
S	WTFGAGAAL	898	906
S	FAMQMAYRF	910	918
S	AEIRASANL	1030	1038
S	VVFLHVTYV	1075	1083
S	KEIDRLNEV	1197	1205
S	VLKGVKLHY	1282	1290

E, envelope, M, membrane, N, nucleocapsid, S, spike.

TABLE 2 | Potential immunogenic $CD8^+$ T-cell epitopes pertaining to SARS-CoV-2.

Protein	*Peptides*	*Start*	*End*
M	RINWITGGIA	72	81
M	VYRINWITGG	70	79
NSP3	DCEEEEFEPS	935	944
NSP2	EHEHEIAWYT	233	242
NSP3	GDCEEEEFEP	934	943
2′-O-ribose methyltransferase (NSP16)	GHFAWWTAF	6983	6991
2′-O-ribose methyltransferase (NSP16)	GHFAWWTAFV	6983	6992
2′-O-ribose methyltransferase (NSP16)	HFAWWTAFV	6984	6992
NSP2	KLNEEIAIIL	468	477
NSP2	LNEEIAIILA	469	478
NSP4	LVPFWITIA	3135	3143
NSP4	LVPFWITIAY	3135	3144
2′-O-ribose methyltransferase (NSP16)	MGHFAWWTA	6982	6990
2′-O-ribose methyltransferase (NSP16)	MGHFAWWTAF	6982	6991
NSP4	PLVPFWITIA	3134	3143
NSP4	TKHFYWFFS	3150	3158
NSP4	VPFWITIAY	3136	3144
NSP4	VPFWITIAYI	3136	3145
S	NVTWFHAIHV	61	70
ORF7b	IMLIIFWFSL	23	32
ORF7b	MLIIFWFSL	24	32

E, envelope, M, membrane, N, nucleocapsid, S, spike, NSP, non-structural protein.

peptides with IC50 less than 50 nM as a cut-off to select strong binders (Wang et al., 2008). Using this selection criterion, we obtained 1478 strong binders restricted by MHC-II alleles. These predicted binders were subjected to antigenicity prediction. Out of the 1478 predicted MHC-II peptides, 831 were found to be antigenic on the basis of a Vaxijen score greater than 0.4. After subjecting these antigenic peptides prediction of Interferon gamma (IFN-γ) secreting peptides, only 304 were found to be positive according to the IFNepitope score. Toxicity prediction reduced this number to 296 (i.e., non-toxic). These 296 peptide sequences were then further subjected to allergenicity prediction, and 194 peptides were found to be non-allergenic, while 102 were allergenic. These 194 peptides can be used as vaccine candidates to elicit helper T-cells ($CD4^+$) (**Supplementary Tables S7, S8**). Additionally, epitope conservancy and population coverage by these epitopes were also determined. Out of these 194 peptides, only three sequences were 100% conserved (S: 2, ORF1ab: 1) within the SARS-CoV sequence, while the total number of sequences with more than 90% conservancy was 78, with a variable degree of conservation with other CoVs (**Supplementary Table S7**).

Prediction of immunogenic CD4 + T-cell epitopes from SARS-CoV-2 proteins using the "CD4 T cell immunogenicity prediction tool" available at the IEDB resulted in 319 immunogenic peptides. Out of these 319 epitopes, 132 were found to be antigenic. Further testing of these peptides for toxicity resulted in 129 peptides where no "non-toxic" peptide was found in ORF10. Among these 129 peptides, 44 were found to be non-allergic (**Supplementary Tables S9, S10**) and, thus, can be safely used for vaccine formulations after testing them further for conservancy and population coverage. The conservancy analysis showed that 19 epitopes were 100% conserved with SARS-CoV sequences, while there were 28 sequences in total that were more than 90% conserved with SARS-CoV (**Supplementary Table S9**).

Likewise, 320 linear B-cell epitopes were predicted from the SARS-CoV-2 proteins. Predicted epitopes varied in length from 111 (maximum) to a single amino acid residue (minimum). Of these epitopes, only 135 were found to be antigenic using Vaxijen. Toxicity prediction of these 135 antigenic peptides resulted in 126 non-toxic and 9 toxic sequences. Allergenicity prediction of these non-toxic peptides showed that only 65 sequences were predicted to be non-allergenic, while the remaining 61 were allergenic. These epitope sequences and their lengths, start and end points in a protein, and conservancies are shown in **Supplementary Table S11**. **Supplementary Table S12** shows the protein-wise distribution of the counts of these epitopes. These 65 sequences were further tested for conservancy with other coronavirus strains (**Supplementary Table S11**).

Of these 65 epitopes, 20 were found to be 100% conserved and 26 sequences (N = 2, S = 1, ORF1ab = 23) were more than 90% conserved with SARS-CoV (**Table 3**). These 20 sequences are located in ORF1ab polyprotein in various regions. One Spike glycoprotein (S) epitope (^{404}GDEVRQIAPGQTGKIA DYNYKLP^{426}) with a length of 23-mer was found to be 91.3% conserved with the SARS-CoV spike protein. For envelope protein, only one sequence (^{57}YVYSRVKNLNSSRVP^{71}) was conserved within SARS-CoV, with 80% conservancy. Nucleocapsid protein (N) had two sequences (^{226}RLNQLESKMS GKGQQQQGQTVTKKSAAEASKKPRQKRTATKA^{267} and ^{276}R RGPEQTQGNFGDQELIRQGTDYK^{299}) that were more than 95% conserved with the SARS-CoV sequence (**Supplementary Table S11**).

The epitopes from ORF1ab polyprotein, with length greater than 9-mer, were found to be variously conserved within ORF1ab polyprotein from the other six viruses. Two sequences (KLQNNELSPVAL and SYKDWSYSGQ) each of length 12-mer and 10-mer were conserved within four other coronavirus strains, while few are conserved within only SARS-CoV. No epitope sequences were found to be conserved within these five coronavirus strains according to our set criteria (**Supplementary Table S11**).

Further, a total of 37 conformational B-cell epitopes were predicted using the Ellipro method (**Supplementary Table S13**). The top 10 sequences of these 37 predicted epitopes had protrusion scores lying between 0.77 and 0.99. A high protrusion index (PI) value means enhanced solvent availability. Among these 10 sequences, 5 sequences belonging to the proteins ORF3a [(M1, D2, L3, F4, M5, R6), (T9, I10, G11, T12, V13, T14, L15)], ORF7b (E39, T40, C41, H42, A43), ORF8 (E92, P93, K94), and ORF10 (R24, N25, Y26) had PI scores above 0.80. The two highest-scoring peptides belonged to ORF3a, with PI scores of 0.99 and 0.94, respectively (**Supplementary Table S13**).

A few recent immunological studies have experimentally validated several epitope sequences and found some to be positive in qualitative/quantitative assays against SARS-CoV-2 proteins,

TABLE 3 | Putative linear B-cell epitopes for SARS-CoV-2 and SARS-CoV.

Protein	Peptides	Start	End	Length
N	RLNQLESKMSGKGQQQQGQTVTKKSAAEASKKPRQKRTATKA	226	267	42
N	RRGPEQTQGNFGDQELIRQGTDYK	276	299	24
S	GDEVRQIAPGQTGKIADYNYKLP	404	426	23
Leader protein (NSP1)	LPVLQV	18	23	6
NSP3	SYKDWSYSGQ	1510	1519	10
NSP3	FPDLNG	1960	1965	6
NSP3	TRQVVNV	2747	2753	7
3C-like proteinase (NSP5)	MAFPSGK	3269	3275	7
3C-like proteinase (NSP5)	YNYEPLTQDH	3500	3509	10
NSP7	VQSKMSD	3858	3864	7
NSP8/NSP9	KLQNNELSPVAL	4138	4149	12
RDRP (NSP12)	PCGTGTSTDV	4413	4422	10
RDRP (NSP12)	TFSNYQHEET	4468	4477	10
RDRP (NSP12)	VAFQTVKPGNFNKDFYDFAVSKGFFKEGSSVEL	4797	4829	33
RDRP (NSP12)	LKYAISAKNR	4936	4945	10
RDRP (NSP12)	KPGGTSSGDATT	5068	5079	12
RDRP (NSP12)	WTETDLTKGP	5192	5201	10
Helicase (NSP13)	TCVGSDNVTDFNAIATCDWTNAGDYILANTCTE	5420	5452	33
Helicase (NSP13)	FEKGDYG	5524	5530	7
Helicase (NSP13)	PAPRTLLTKGTLEPE	5730	5744	15
Helicase (NSP13)	LYDKLQ	5905	5910	6
Helicase/3′-5′ Exonuclease	RNVATLQAENVTG	5919	5931	13
3′-5′ Exonuclease (NSP14)	MYKGLPW	6078	6084	7
3′-5′ Exonuclease (NSP14)	GFTGNLQSNHDLYCQVHGNAHVA	6173	6195	23
2′-O- ribose methyltransferase (NSP16)	DKGVAP	6873	6878	6
2′-O- ribose methyltransferase (NSP16)	IQLSSYSLFDMSKFPLKLRG	7035	7054	20

specifically spike glycoprotein (Poh et al., 2020; Smith et al., 2020; Yi et al., 2020; Yuan et al., 2020).

Poh et al. (2020) have found 2 linear B-cell epitopes (S14P5 and S21P2) in the spike protein of SARS-CoV-2 and our predicted linear B-cell epitope sequences, namely- ^{555}**SNKKFLPF**^{562} and ^{807}PD**PSKPSK**^{814}, in the spike protein of SARS-CoV-2 mapped on these two experimentally validated epitopes. These epitopes were found to be antigenic and non-toxic but allergenic in nature.

A study published in Science by Yuan and group found a conformational B-cell epitope in the receptor-binding domain that was highly conserved between SARS-CoV-2 and SARS-CoV (Yuan et al., 2020). We have also predicted a linear B-cell epitope that is a part of this discontinuous epitope with the sequence ^{369}**YNSASFSTFKCYGVSPTK**LND**L**CFT^{393}. This epitope was found in our analysis to be antigenic, non-toxic, and non-allergenic, having 84% sequence conservancy with SARS-CoV.

Similarly, Yi et al. (2020) also found some key residues in the spike protein of SARS-CoV-2 that interact with ACE2 as well as with neutralizing antibodies. These residues mapped on our predicted linear B-cell epitope ^{369}**YNSASFSTFKCYGVSPTK**LND**L**CFT^{393} (mentioned above).

Trevor et al., found nine MHC-I-restricted T-cell epitopes in the spike protein of SARS-CoV-2 (Smith et al., 2020). Out of these nine epitopes, three were found to match with our predicted epitopes. "**VLSFELLHA**" mapped on one of the epitopes (V**VLSFELLHA**PATVC) and was found to be antigenic, non-toxic, and non-allergenic in our analysis. In the same way, **"VVFLHVTYV"** was predicted to be positively antigenic, non-toxic, and non-allergenic and completely mapped on an epitope (PHG**VVFLHVTYV**PAQ) found in the above-mentioned study. These two sequences can be used as vaccine candidates to elicit the adaptive arm of the immune system and provide protection against SARS-CoV-2. Epitope sequence "KIAD**YNYKL**," predicted as a positive epitope by this study, also had a few residues matching with an experimentally confirmed epitope (**YNYKL**PDDFTGCVIA). However, it was found to be allergenic in our study.

Population Coverage Analysis

The T-cell epitopes selected following the conservancy analysis were used to compute population coverage. We used the population coverage tool offered by the IEDB (see text footnote 14) to compute the population covered by predicted MHC-I, MHC-II binders, and CTL epitopes from SARS-CoV-2 (Bui et al., 2006).

The maximum population coverage of predicted MHC-I binders (which are also antigenic, non-toxic, and non-allergenic) was found for the European population (97.71%), which was followed by North America, West Indies, West Africa, Southeast Asia, Northeast Asia, North Africa, Oceania, South Africa, South Asia, East Africa, South America, Southwest Asia, Central Africa,

and Central America, with predicted population coverages (PPC) of 97.48, 96.96, 92.96, 92.68, 92.61, 92.46, 91.64, 88.81, 88.78, 86.85, 85.18, 84.32, 83.82, and 7.76%, respectively, as shown in **Supplementary Table S14**.

The highest PPC for CTL epitopes was also found for the European population (95.66%), which was immediately followed by the North American population (87.54%). The PPC for Northeast Asia, including China (the area of COVID-19 outbreak), covered by these epitopes was quite low (65.65%) as compared to the high PPC (92.61%) for MHC-I binders for the same region. For MHC-II binders, the highest PPC was observed for the North American population (99.99%) closely followed by the European population (99.92%). Here, the area of Northeast Asia also had a high estimated PPC (93.81%). It is to be noted that the estimated PPC for European countries including Italy (most effected by COVID-19 along with China, United States, and Spain to date) provided by our predicted epitopes was very high (>99%) (**Supplementary Table S14**).

T-Cell and B-Cell Epitopes of All Coronaviruses

The search for T-cell and B-cell epitopes from all global coronaviruses was performed in the IEDB, which harbored details for the following coronaviruses: Alphacoronavirus 1, Avian coronavirus, Betacoronavirus 1, Coronavirus HKU15, HCoV-229E, MERS-CoV, Murine coronavirus, Porcine epidemic diarrhea virus, SARS-CoV, and Swine acute diarrhea syndrome-related coronavirus. We obtained 320 positive T-cell epitopes, with 778 T-cell assays related to these epitopes. Similarly, 663 positive B-cell epitopes with 1568 B-cell assays were found in IEDB. Of these 663 epitopes, 582 were linear and 81 were conformational. The conservancy analysis of T-cell and B-cell epitopes from these coronaviruses with SARS-CoV-2 proteins showed that 41 unique T-cell and 83 linear B-cell epitopes were 100% conserved within SARS-CoV-2. Only Humans (*Homo sapiens*) and various experimental mice (*Mus musculus*) were found as hosts for these coronaviruses in the case of T-cell epitopes. However, in the case of linear B-cell epitopes from other coronaviruses that shared 100% conservancy with SARS-CoV-2, various animals such as the Formosan rock macaque (*Macaca cyclopis*), Guinea pig (*Cavia porcellus*), and Rabbit (*Oryctolagus cuniculus*) were also observed as hosts as well as *Homo sapiens* and *Mus musculus*.

Cross-Protective Epitopes (CPEs) Between SARS-CoV and SARS-CoV-2

At a time when a vaccine is urgently required against SARS-CoV-2, the non-availability of epitope information for it is a shortcoming that may lengthen the vaccine development process. To help the researchers in developing a SARS-CoV-2 vaccine, we sought to identify the cross-protective epitopes (CPEs) and unique epitopes (UE) based on antigenic similarities and differences between SARS-CoV epitopes and SARS-CoV-2 protein sequences. For this, we extracted 119 T-cell and 405 linear B-cell epitope sequences of SARS-CoV (ID: 694009) available on the IEDB. These 119 T-cell epitopes were dispersed in 51 and 68 MHC class I and class II alleles, respectively. The conservancy analysis to find cross-protective epitopes was performed by mapping SARS-CoV T- and B-cell epitopes on SARS-CoV-2 proteins. Of the 119 T-cell epitopes, 27 potential cross-protective epitopes were found with 100% conservancy (no mutation) distributed in four different proteins (N: 13, S: 12, ORF1ab: 1, and M: 1) of SARS-CoV-2. Altogether, 75 T-cell epitopes of SARS-CoV were found with high sequence identity (>80%) with SARS-CoV-2. On the other hand, 13 sequences had moderate similarity (>70% but <80%) and 29 had low similarity (<70%) and can be considered as unique in SARS-CoV-2 (**Supplementary Table S15**).

We also checked the IFNepitope score (for MHC-II epitopes), toxicity, and allergenicity of these experimentally confirmed T-cell epitopes of SARS-CoV and their corresponding sequences in SARS-CoV-2. Among the 27 epitopes that were 100% conserved, 14 were predicted to be non-toxic and non-allergenic (**Table 4**). Out of the 7 MHC-II restricted T-cell epitopes (100% conserved), only 2 were found to have positive IFNepitope scores, and only one (^{306}AQFAPSASAFFGMSR^{320}) was found to be non-toxic and non-allergenic. **Supplementary Table S15** lists T-cell epitopes of SARS-CoV with conservancy with SARS-CoV-2 that fulfill other criteria [IFNepitope (for MHC II), Non-Toxic, Non-Allergenic] that can be used as vaccine candidates to provide cross-protection against each other.

In parallel, the mapping of linear B-cell epitopes of SARS-CoV showed that out of 405 epitopes, 83 were 100% conserved in SARS-CoV-2 proteins (E: 1, ORF1ab: 1, M: 6, N: 32, and S: 43). Comprehensively, there were 237 epitopes with sequence identity >80% (**Supplementary Table S16**). Toxicity and allergenicity prediction of SARS-CoV epitopes resulted in 45 non-toxic and non-allergenic sequences that were 100% conserved with SARS-CoV-2 proteins. These 45 shared epitopes between SARS-CoV and SARS-CoV-2 can provide cross-protection against each other and can be utilized as potent linear B-cell epitopes to elicit humoral immunity (**Table 5**).

Overall, an immuno-informatics-driven methodology was implemented to discover the B-cell (linear and conformational) and T-cell ($CD8^+$ and $CD4^+$) epitopes, which can help researchers at the initial stage of the design of vaccine against SARS-CoV-2. With no experimentally confirmed epitopes of SARS-CoV-2 to date, we sought to address potential epitopes using various computational tools. We also considered various other properties, neglecting which may destroy the purpose of the development of a vaccine against SARS-CoV-2, such as antigenicity, toxicity, and allergenicity.

Some parallel studies are available that have identified different epitope components of SARS-CoV-2 through bioinformatics predictions (Ahmed et al., 2020; Baruah and Bose, 2020; Grifoni et al., 2020; Lucchese, 2020; Qiu et al., 2020). Grifoni et al., mapped experimentally confirmed epitopes of SARS-CoV on SARS-CoV-2 and predicted new epitope sequences as well (Grifoni et al., 2020). Ahmed et al., also mapped experimentally confirmed epitopes of SARS-CoV on SARS-CoV-2 and analyzed the population coverage for T-cell epitopes to find epitopes for vaccine formulation (Ahmed et al., 2020). Qui T. et al., searched for cross-protective epitopes on Spike protein of SARS-CoV-2

TABLE 4 | Potential cross-protective T-cell epitopes (vaccine candidates) against SARS-CoV-2 and SARS-CoV.

Protein	MHC type	SARS-CoV-2 Epitopes	Start	End	Length	Allergenicity
S	MHC-I	VNFNFNGL	539	546	8	Allergen
N	MHC-I	ILLNKHID	351	358	8	Allergen
S	MHC-I	ALNTLVKQL	958	966	9	Non-Allergen
N	MHC-I	ALNTPKDHI	138	146	9	Allergen
S	MHC-I	FIAGLIAIV	1220	1228	9	Non-Allergen
N	MHC-I	GMSRIGMEV	316	324	9	Non-Allergen
N	MHC-I	ILLNKHIDA	351	359	9	Allergen
N	MHC-I	LALLLLDRL	219	227	9	Non-Allergen
S	MHC-I	LITGRLQSL	996	1004	9	Allergen
N	MHC-I	LLLDRLNQL	222	230	9	Allergen
N	MHC-I	LQLPQGTTL	159	167	9	Allergen
S	MHC-I	NLNESLIDL	1192	1200	9	Allergen
S	MHC-I	RLNEVAKNL	1185	1193	9	Allergen
ORF1ab	MHC-I	VLAWLYAAV	3467	3475	9	Non-Allergen
S	MHC-I	VLNDILSRL	976	984	9	Non-Allergen
S	MHC-I	VVFLHVTYV	1060	1068	9	Non-Allergen
M	MHC-I	TLACFVLAAV	61	70	10	Non-Allergen
N	MHC-I	MEVTPSGTWL	322	331	10	Non-Allergen
N	MHC-I	RRPQGLPNNTASWFT	40	54	15	Allergen
N	MHC-II	AQFAPSASAFFGMSR	305	319	15	Non-Allergen
N	MHC-II	SPRWYFYYLGTGPE	105	119	15	Non-Allergen
N	MHC-II	VILLNKHIDAYKTFP	350	364	15	Allergen
S	MHC-II	GAALQIPFAMQMAYRF	891	906	16	Non-Allergen
S	MHC-II	MAYRFNGIGVTQNVLY	902	917	16	Non-Allergen
S	MHC-II	QALNTLVKQLSSNFGAI	957	973	17	Non-Allergen
N	MHC-I	LLNKHIDAYKTFPPTEPK	352	369	18	Allergen
S	MHC-II	QLIRAAEIRASANLAATK	1011	1028	18	Allergen

based on similarity with epitopes of SARS-CoV (Qiu et al., 2020). Lucchese et al., addressed pentapeptides of SARS-CoV-2 proteins absent in human as vaccine candidates (Lucchese, 2020). Bose et al., identified T- and B-cell epitopes in spike protein of SARS-CoV-2 using an immunoinformatics method (Baruah and Bose, 2020).

Our work is different from other studies in several aspects and gives various new insights that are important for designing vaccine formulations against SARS-CoV-2. The most important difference is that we are providing a unifying online platform for easy, free, and direct access to components to assist researchers. We have not limited our study to a selected few but have performed a comprehensive analysis of all proteins of SARS-CoV-2, specifically the structural proteins, to find vaccine candidates.

We performed antigenicity, toxicity, and allergenicity prediction of our addressed epitope sets since these are important considerations in vaccine formulation. For the MHC-II-restricted epitopes predicted in our study, we have also predicted the IFN-gamma-inducing ability of these peptides. We have performed cross-conservancy analysis of predicted epitopes with other coronaviruses causing diseases in Humans. To the best of our knowledge, no study has performed these analyses on SARS-CoV-2 proteins.

Our study suggests several epitopes as probable vaccine candidates on the basis of antigenicity, toxicity, and allergenicity along with IFN-gamma-inducing properties for MHC-II-restricted epitopes. The epitope mapping on the proteins of other human-infecting coronavirus strains showed conservancy to SARS-CoV to variable degrees. We found 169, 2, 19, and 20 CTL, immunogenic $CD8^+$, immunogenic $CD4^+$, and B-cell epitopes, respectively, with 100% sequence conservancy within SARS-CoV, which can be used as potent vaccine candidates against both of the viruses. However, very few sequences were found to be conserved with the other five coronaviruses, highlighting the fact that SARS-CoV-2 is quite different from these human-infecting viruses. This finely selected list of predicted epitopes of SARS-CoV-2 can be tested in future studies for the elicitation of immune response for their use as vaccine candidates. We have predicted T-cell epitopes in order to cover the Chinese ethnicity as well as the majority of the population around the world.

Several studies have highlighted the importance of the adaptive arm of the immune system (i.e., T-cells and B-cells) in providing protection against SARS-CoV (Wang et al., 2004; Xu and Gao, 2004; Chen et al., 2010; Channappanavar et al., 2014; Liu et al., 2017). We have identified SARS-CoV T-cell and B-cell epitopes with 100% conservancy in SARS-COV-2 proteins. These are cross-protective and can be used for designing a vaccine against SARS-CoV-2. A total of 27 T-cell epitopes of SARS-CoV were found that were fully conserved in different proteins (N: 13, S: 12, ORF1ab: 1, and M: 1) of SARS-CoV-2. We also checked peptide sequences of SARS-CoV-2 proteins that

TABLE 5 | Potent cross-protective B-cell epitopes (vaccine candidates) against SARS-CoV-2 and SARS-CoV.

Protein	SARS-CoV-2 Epitopes	Start	End	Length
E	RVKN	61	64	4
M	LEQWNLVIGFLFL	17	29	13
M	PKEITVATSRTLSYYKL	165	181	17
M	GRCDIKDLPKEITVATSR	157	174	18
N	GSFCTQLN	278	285	8
N	LPQRQKKQ	382	389	8
N	SQASSRSS	180	187	8
N	TFPPTEPK	362	369	8
N	LPQGTTLPKG	161	170	11
N	GFYAEGSRGGSQASS	170	184	15
N	GSRGGSQASSRSSSR	175	189	15
N	KTFPPTEPKKDKKKK	361	375	15
N	TTLPKGFYAEGSRGG	165	179	15
N	YKTFPPTEPKKDKKK	360	374	15
N	FFGMSRIGMEVTPSGTW	314	330	17
N	KHWPQIAQFAPSASAFF	299	315	17
N	QFAPSASAFFGMSRIGM	306	322	17
N	PKGFYAEGSRGGSQASSR	168	185	18
N	QLPQGTTLPKGFYAEGSR	160	177	18
N	KHIDAYKTFPPTEPKKDKKK	355	374	20
N	VTQAFGRRGPEQTQGNFGDQ	270	289	20
N	QLPQGTTLPKGFYAEGSRGGSQ	160	181	22
S	AMQMAYRF	899	906	8
S	GAGICASY	667	674	8
S	KGIYQTSN	310	317	8
S	DDSEPVLKGVKLHYT	1259	1273	15
S	DKYFKNHTSPDVDLGD	1153	1168	16
S	AISSVLNDILSRLDKVE	972	988	17
S	EAEVQIDRLITGRLQSL	988	1004	17
S	EELDKYFKNHTSPDVDL	1150	1166	17
S	GAALQIPFAMQMAYRFN	891	907	17
S	IRQGTDYKHWPQIAQFA	292	308	17
S	KEIDRLNEVAKNLNESL	1181	1197	17
S	MAYRFNGIGVTQNVLYE	902	918	17
S	PELDSFKEELDKYFKNH	1143	1159	17
S	PFAMQMAYRFNGIGVTQ	897	913	17
S	QALNTLVKQLSSNFGAI	957	973	17
S	RLITGRLQSLQTYVTQQ	995	1011	17
S	SLQTYVTQQLIRAAEIR	1003	1019	17
S	TVYDPLQPELDSFKEEL	1136	1152	17
S	CKFDEDDSEPVLKGVKLHYT	1254	1273	20
S	EIDRLNEVAKNLNESLIDLQELGKYEQY	1182	1209	28
S	EIDRLNEVAKNLNESLIDLQELGKYEQY	1182	1209	29
S	DSFKEELDKYFKNHTSPDVDLGD ISGINASVV	1146	1177	32
S	ISGINASVVNIQKEIDRLNEVAK NLNESLIDLQELGKYEQYI	1169	1210	42

were found with variable levels of conservancy with SARS-CoV epitopes and predicted their antigenicity, toxicity, IFN-gamma-secreting ability, and allergenicity. We found one MHC-II-restricted epitope, namely, ^{306}AQFAPSASAFFGMSR^{320}, present in nucleocapsid of SAR-CoV that was 100% conserved within SARS-CoV-2 and was predicted to be antigenic, non-toxic, IFN-gamma-inducing and non-allergenic. Hence, this epitope sequence can be incorporated in designing a vaccine to provide cross-protection against SARS-CoV and SARS-CoV-2. We also found 45 shared linear B-cell epitopes between SARS-CoV and SARS-CoV-2 that were antigenic, non-toxic, and non-allergenic that can provide cross-protection against each other and can be utilized as potent vaccine candidates to elicit humoral immunity.

We expect that this study may help researchers in developing an inexpensive epitope-based vaccine against SARS-CoV-2 that may provide immunity to the entire world's population.

siRNAs and miRNAs

RNA interference-based silencing of viral genes provides an excellent alternative therapeutic tool. For this, we also explored and provided a compilation of putative efficient siRNAs against all of the genes of SARS-CoV-2. In total, 166 potent siRNAs with more than 60% inhibition were identified using the VIRsiRNApred algorithm. The different siRNAs targeting different genes of SARS-CoV-2 are provided in **Supplementary Table S17**. Correspondingly, 1163 putative siRNAs with efficacy scores equal to or more than 1 were also recognized utilizing the desiRm method and are provided on the server. For all of the siRNAs, the sense–antisense sequence, gene target, start-end, efficacy scores, immunomodulatory potential, and off-target information are provided on the CoronaVR resource. Additionally, we have also identified SARS-CoV-2 pre-miRNAs and mature miRNAs. Overall, 50 pre-miRNAs were identified, with a pair of mature miRNAs (5p and 3p). Complete information on precursor (hairpin) sequence, precursor length, location (start-end), genomic region, mature-miRNA sequence, GC content, etc., is provided (**Supplementary Table S18**).

sgRNA-Based Genome Editing

Based on our analysis, 64 putative efficient sgRNAs were identified for SARS-CoV-2. Complete information like sgRNA sequences (5′-3′), PAM, start and end positions of the sgRNAs in the genome, GC%, and predicted sgRNA efficiency (%) is displayed in tabular format. This analysis will certainly help the scientific community to identify potential CRISPR targets and to design efficient sgRNAs against SARS-CoV-2 prior to experimental procedures. Highly efficient sgRNAs targeting SARS-CoV-2 are provided in **Supplementary Table S19**.

Molecular Diagnostic Primers

The literature was searched in PubMed using different keywords, i.e., "coronavirus," "homo-sapiens/humans," and "primers*," and a total of 185 papers were retrieved. Overall, 198 primer sets specific for different strains of CoVs were obtained. Of these, 7 primer pairs are specific for SARS-CoV-2, 47 are for SARS-CoV, 25 are for MERS-CoV, and 107 are for the different HCoVs (229E-45, OC43-28, NL63-23, and HKU1-9). Additionally, we also identified three universal primer pairs, 6 sets of primers for beta-CoVs (SARS-CoV, MERS-CoV, OC43, and HKU1), and 2 primer sets specific to the alpha-CoVs (229E and NL63). These primers are specific for the particular genes, and some are applicable for the whole genome of the CoVs. Among all of them, 67 primers

belong to the N gene, 6 primers are for gene E, 14 belong to the S gene, 9 primers are for gene M, 27 primers are specific for RdRp, 3 primer sets are for the UTR region, 17 are for ORF1a, 13 belong to ORF1b, 13 are for orf1ab, and 1 primer set is for ORF8.

Furthermore, we also designed specific primers for the different genes of SARS-CoV-2 using the Primer Design-M tool. In total, 21 primer sets were designed that are specific to the individual genes. Among these primer pairs for each gene, i.e., M, N, ORF3a, ORF6, ORF7a, ORF7b, and ORF10, 4 belong to ORF8 and 3 primer sets were designed for the S gene.

Glycosylation in SARS-CoV-2

We also explored glycosylation sites in SARS-CoV-2. For this, three types of glycosylation sites, namely, C-linked, N-linked, and O-linked, were deduced. In total, 130 sites, i.e., 52 N-glycosylated (N-Gly) sites, and 78 O-glycosylated (O-Gly) sites were predicted. However, we could not find any C-mannosylated sites in SARS-CoV-2.

The protein-wise N-linked glycosylation sites are as follows M (1), E (2), S (17), ORF6 (1), ORF7b (1), ORF8 (1), N (2), nsp2 (3), Papain-like proteinase (8), Proteinase 3CL-PRO (2), nsp6 (1), nsp9 (1), nsp10 (2), Helicase (3), Guanine-N7 methyltransferase (3), Uridylate-specific endoribonuclease (2), and 2′-O-methyltransferase (2). In contrast, there are no single N-glycosylation sites found in ORF3a, ORF7a, ORF10, Host translation inhibitor nsp1, nsp4, nsp7, nsp8, and RdRp. Likewise, protein-wise O-linked glycosylation sites are as follows: S (3) and N (47), Host translation inhibitor nsp1 (1), Papain-like proteinase (14), nsp9 (1), RdRp (2), Helicase (6), Guanine-N7 methyltransferase (3), and Uridylate-specific endoribonuclease (1). The remaining proteins, viz., ORF3a, E, M, ORF6, ORF7a, ORF7b, ORF8, ORF10, nsp2, nsp4, Proteinase 3CL-PRO, nsp6, nsp7, nsp8, nsp10, and 2′-O-methyltransferase, do not contain any O-linked glycosylation sites.

Phylogenomics

The 48 viral genomes and their corresponding proteome that were selected for the construction of the phylogenetic tree included 36 SARS-CoV-2 strains, and the remaining 12 were from SARS coronavirus, MERS, and different HCoVs strains, viz., NL63, HKU1, 229E, and OC43. A similar pattern of positioning of the viral taxa has been observed in the previous reports (Benvenuto et al., 2020a,b; Malik et al., 2020), where all of the SARS-CoV-2 strains were clustered together, indicating their uniqueness and identity when compared with the previously reported strains (**Supplementary Figures S1, S2**).

Codon Usage and Bias Analysis

We analyzed the nucleotide composition, amino acid numbers, number of codons, relative synonymous codon usage (RSCU), rare codons, codon context, effective number of codons (ENC), and codon adaptation index (CAI) for the different genes of SARS-CoV-2. The nucleotide composition of all of the coding regions in SARS-CoV-2 revealed that the most and least frequent bases are T and G, respectively. The nucleotide frequencies were T > A > C > G (**Supplementary Table S20**). Also, the same frequency is observed for nucleotides at the third position (NT3s) of a codon. This shows that AT% > GC% in the SARS-CoV-2 genome (**Supplementary Table S20**). Further, codon numbers and RSCU were analyzed. This gives the ratio of expected to observed frequencies of synonymous codon usage by amino acids. An RSCU value of 1 indicates no bias in codon usage, whereas RSCU values <1 or >1 indicate negative and positive codon usage bias (Sheikh et al., 2020). From the RSCU values for different coding regions, the most preferred (RSCU $\geq$ 1.5) and the least favored codons (RSCU $\leq$ 0.5) are identified in **Supplementary Table S21**. A list of codons and RSCUs values for each coding region, i.e., ORF1ab, ORF1a, ORF3a, ORF6, ORF7a, ORF7b, ORF8, ORF10, E, M, N, and S are provided on the web resource. The analysis showed that U3s and A3s were the most recurrent nucleotides in the represented (preferred) codons and that C3s and G3s were the least frequent in all coding regions. Furthermore, gene-wise rare codons are also shown in a histogram. Simultaneously, gene-wise codon context analysis is also performed using Anaconda software, which provides the association between two codons, and the color scale indicates the preferred (green color with residual value more than +3), rejected (red color with residual value more than −3), and codon context with no bias (black color with residual values −3 to +3) codon pairs. The codon context for all of the coding regions is also provided on the server.

Moreover, codon usage bias is also deduced by determining the effective number of codons (ENC values) for different coding regions (**Supplementary Table S22**). ENC values range between 20 and 61. The higher ENC values indicate low codon bias, which indicates that more synonymous codons are used for amino acids (Chen et al., 2017). ENC values for different regions except ORF7b are greater than 40, which also shows low codon usage bias in SARS-CoV-2. In order to look into the relative adaptiveness of SARS-CoV-2 to its host, the codon adaptation index (CAI) was also calculated (**Supplementary Table S22**). CAI values range from 0 to 1, where 1 indicates that the gene always uses the most frequently used synonymous codons in the reference set (Castells et al., 2017). The mean CAI value for all coding regions is 0.686, which is greater than 0.5 and indicates moderate adaptability of SARS-CoV-2 to its host.

We have assessed all of the coding regions of SARS-CoV-2 for codon usage patterns, bias, and adaptability to the host. SARS-CoV-2 showed low GC content, like other members of the *Coronaviridae* family, such as SARS-CoV (Zhao et al., 2008), MERS-CoV (Chen et al., 2017), and BCoV (Castells et al., 2017). The RSCU values for each coding region in SARS-CoV-2 showed that almost all preferred codons ended with Us and As at the 3rd position of synonymous codons, whereas the least preferred ended with Gs and Cs at the 3rd position of synonymous codons. This showed that codon usage bias exists. The mean ENC value (46.845) of all coding regions in SARS-CoV-2 is greater than 40, which indicates low codon usage bias. This is consistent with previous studies on other SARS viruses like BCoV (mean ENC = 43.78), SARS-CoV (ENC = 48.99), Avian coronavirus Infectious bronchitis virus (ENC = 42.79), and Porcine epidemic diarrhea virus (ENC = 47.91) (Castells et al., 2017). The low codon usage bias indicates that SARS-CoV-2 might be able to use many synonymous codons to code for a single amino acid,

which can be helpful in better survival and adaptability of a virus to its host. Further, to gain insight into the adaptation, the codon adaptation index (CAI) for each coding region was calculated in relation to the codon usage of its host, i.e., *Homo sapiens*. The mean CAI value of 0.686 showed better adaptability of SARS-CoV-2 to its host, *Homo sapiens*.

Structural Analysis and Interpretation of SARS-CoV-2 Proteins

In this analysis, six different important proteins of SARS-CoV-2, i.e., RNA-dependent RNA polymerase (RdRp), Helicase, Spike (S), Envelope (E), Nucleocapsid (N), and membrane (M) were structurally analyzed and compared against the other human-infecting CoVs, namely, SARS-CoV, MERS-CoV and other HCoVs (OC43, 229E, NL63, and HKU1). The structures of these proteins from different CoVs along with all of the SARS-CoV-2 proteins were predicted (**Supplementary Tables S23, S24**). The templates used for the structure prediction are also provided. A structural comparison of these proteins is shown in **Supplementary Figure S3**. Additionally, root mean square deviation (RMSD) values for all of the protein comparisons are provided in **Supplementary Table S25**. Among these proteins, we have mainly focused on the two vital drug targets, viz., RdRp and S proteins.

RNA-Dependent RNA Polymerase (RdRp)

RdRp proteins of SARS-CoV-2 and SARS-CoV share a remarkable 96.4% sequence identity, and other strains of CoVs, i.e., MERS, HKU1, OC43, NL63, and 229E and share 71, 67, 66, 59, and 58%, respectively (**Supplementary Figure S4**). RdRp involves a very large and deep groove as an active site for the polymerization of RNA (**Supplementary Figure S3**). Higher sequence conservation between RdRp enzymes makes it very likely that any potent agents developed for SARS-CoV and other strains of CoV RdRp will exhibit equally good potency and efficacy against SARS-CoV-2 RdRp. Further, **Figure 3** shows a protein structure comparison of the RdRp of SARS-CoV-2 with SARS (**Figure 3A**) and seven different strains (**Figure 3B**) of coronavirus along with depictions of functional domains (A-G). **Figure 3C** shows the conservation and variation among different RdRp motifs of CoVs. SARS-CoV shows higher structural similarity with SARS-CoV-2 with a lower RMSD (Root Mean Square Deviation) value (0.005), while OC43 shows the highest divergence, with a RMSD of 0.122 (**Supplementary Table S25**).

Membrane (M) Protein

Membrane (M) proteins represent the major protein component of the viral envelope. During viral assembly, M proteins play a very essential role by interacting with all of the other structural proteins. Its length ranges from 217 to 270 amino acid residues in most CoVs (Perrier et al., 2019). M proteins of SARS-CoV-2 and SARS-CoV share a remarkable 90% sequence identity, and other strains of CoVs, i.e., MERS, HKU1, OC43, NL63, and 229E share 42, 36, 40, 31, and 30%, respectively (**Supplementary Figure S5**). M protein contains three membrane-spanning hydrophobic segments, a small N-terminal domain situated outside the virion, and a large C-terminal domain that makes up half of the protein inside the virion. M proteins of some alphacoronaviruses contain an additional hydrophobic segment that functions as a signal peptide (de Haan and Rottier, 2005).

Envelope (E) Protein

Envelope (E) protein of coronavirus is a small, integral membrane protein containing 76 to 109 amino acids that are involved in assembly, budding, envelope formation, and pathogenesis in the virus life cycle. The E proteins of SARS-CoV-2 and SARS-CoV share a remarkable 94% sequence identity, and other strains of coronavirus MERS, HKU1, OC43, NL63, and 229E share 36, 31, 31, 18, and 27%, respectively (**Supplementary Figure S6**).

Helicase Protein

The unwinding of the double-stranded oligonucleotides into the single-stranded form using ATP during the replication cycle of the coronavirus is carried out by the enzyme helicase. Helicase proteins of SARS-CoV-2 and SARS-CoV share 99.83% sequence identity, and other strains of CoVs, i.e., MERS, HKU1, OC43, NL63, and 229E, share 72, 65, 68, 61, and 60%, respectively (**Supplementary Figure S7**). Structural conservation of these helicase proteins from different CoVs is also shown in **Supplementary Figure S3**. Helicase carries out the unwinding of nucleic acids during replication, recombination and DNA repair and is also involved in other biological processes, like movement of Holliday junctions, chromatin remodeling, displacement of proteins from nucleic acid, catalysis of nucleic acid conformational changes, and several aspects of RNA metabolism and mitochondrial gene expression (Adedeji and Lazarus, 2016). As the helicases of different coronaviruses are very homologous, helicase inhibitors are good and reliable anti-CoV treatment options. The helicase inhibitors can be categorized into two groups depending on their mechanism of action. Bananins and 5-hydroxychromone derivatives come under the first class of inhibitors, which inhibit viral replication *in vitro* by preventing the unwinding and ATPase activity of SARS-CoV helicase (Tanner et al., 2005; Kim et al., 2011). The second class of inhibitors includes those inhibitors that inhibit the unwinding but not the ATPase activity of helicase of CoV (Zumla et al., 2016).

Nucleocapsid (N) Protein

This is a protein with numerous activities. Packaging of the viral genome into a helical ribonucleocapsid (RNP) is done by the nucleocapsid phosphoprotein. It plays a fundamental role during viral self-assembly. The suppression of RNA silencing and RNA interference that is triggered by either short hairpin RNAs or siRNAs is done by the N protein. The SARS-CoV-2 N protein is a phosphoprotein of 419 amino acids, sharing 90% sequence identity with the N protein of SARS-CoV. It shows a sequence identity of 38, 36, 48, 38, and 28% with the 229E, HKU1, MERS, OC43, and NL63 strains, respectively (**Supplementary Figures S3, S8**). N protein consists of two separate domains, an N-terminal domain (NTD) and a C-terminal domain (CTD), which are capable of binding to RNA *in vitro* via different mechanisms (Chang et al., 2006; Hurst et al., 2009). It also binds

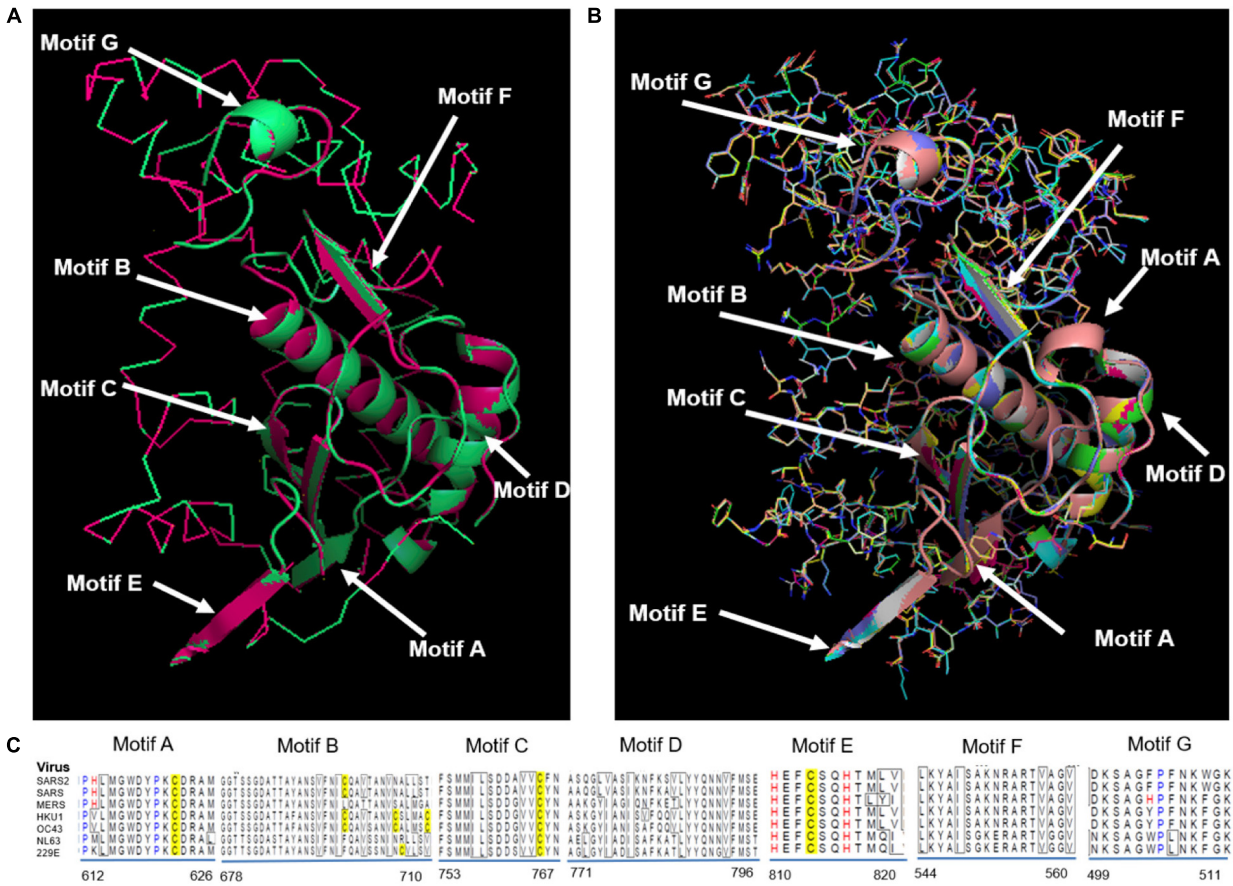


FIGURE 3 | Structural comparison of RdRp. **(A)** SARS-CoV-2 RdRp with SARS-CoV RdRp, **(B)** SARS-CoV-2 compared with SARS-CoV, MERS-CoV, HKU1, OC43, NL63, and 229E. Different functional domains (A-G) of RdRp marked on structure, **(C)** Alignment showing conservation and variation among different motifs of RdRp from distinct CoVs.

to nsp3 and M protein, nsp3 being the key component of the replicase complex (Sturman et al., 1980; Hurst et al., 2009, 2013). These protein interactions are likely to help in the packaging of the encapsulated genome into viral particles (Fehr and Perlman, 2015). Previous studies also show that N protein has been widely used as a diagnostic target of SARS-CoV. Viral N protein is considered to be a genetically stable protein, which is a primary criterion for selecting an efficient drug target candidate. It is even a therapeutic target in antiviral therapy due to its role in pathogenicity inside the cell (Chang et al., 2014).

Spike (S) Glycoprotein

Surface glycoprotein or Spike (S) is a major immunogenic antigen of CoVs that is essential for interactions between a virus and host cell receptor, i.e., angiotensin-converting enzyme 2 (ACE2) and on S protein priming by a cellular protease, i.e., TMPRSS2 (Hoffmann et al., 2020). It has also been found that both SARS-CoV-2 and SARS-CoV use a common receptor, ACE2, for entry, and this is important for understanding the transmissibility and pathogenesis of SARS-CoV-2 (Hoffmann et al., 2020). Further, it is also estimated that SARS-CoV-2 S protein may have high binding affinity toward Human ACE2 (Smith et al., 2020; Zhang H. et al., 2020). In order to activate membrane fusion, virus entry, and syncytium formation, cleavage at the S1-S2 junction is necessary, and it undergoes structural rearrangement (Chan et al., 2015). When receptor-binding domain (RBD) of S1 subunit attaches to the host cell receptors, it causes conformational changes in the S2 subunit, which ultimately leads to the fusion of the viral and the cell membrane by bringing them into close proximity (Lu et al., 2014; Wrapp et al., 2020). Spike glycoprotein can be an ideal target for vaccine and antiviral development due to its role in receptor binding and membrane fusion. Various previous studies summarize the development of SARS vaccines based on the spike protein (Casais et al., 2003; Jiang et al., 2005; Roper and Rehm, 2009; Du et al., 2010). Various ideas and strategies (live-attenuated SARS-CoV, killed SARS-CoV, DNA vaccines, and viral-vectored vaccines) that have been used to develop vaccines against animal-CoVs could be used to develop SARS-CoV-2 vaccines as well. Additionally, a TMPRSS2 inhibitor may block the entry of the virus and might constitute a treatment option (Casais et al., 2003; Jiang et al., 2005; Roper and Rehm, 2009; Du et al., 2010; Morse et al., 2020).

S proteins of SARS-CoV-2 and SARS-CoV share 75% sequence identity, and other strains of CoVs, MERS, HKU1, OC43, NL63,

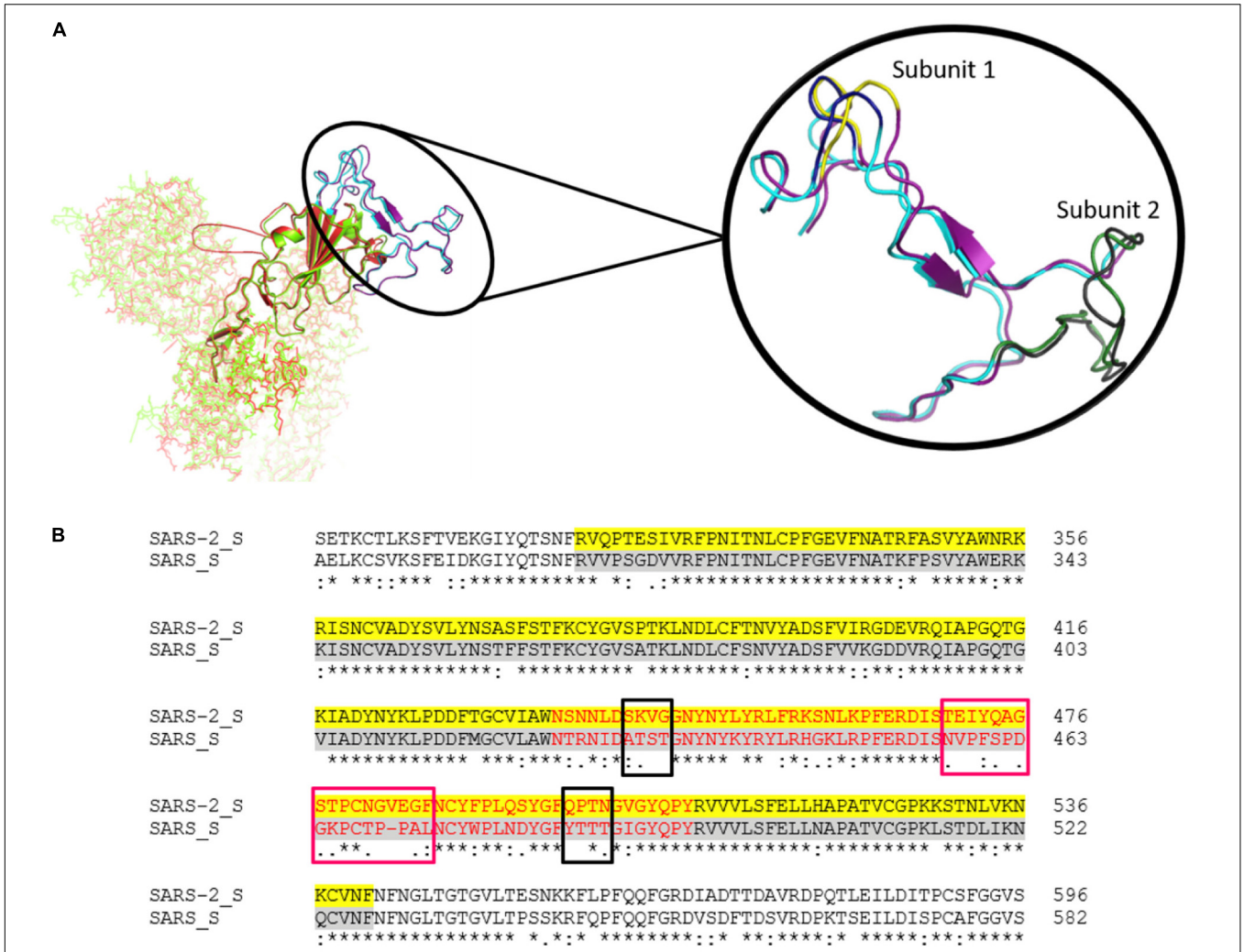


FIGURE 4 | (A) The Spike protein structure of SARS-CoV-2 (green) compared to SARS-CoV (red). The RBD is represented by a cartoon, while the rest of the protein is represented by sticks. The highlighted part is the comparison of the RBM of SARS-CoV-2 (Purple) and SARS-CoV (Cyan). Magnified view of RBM shows both subunits. Subunit 1 of SARS-CoV-2 and SARS-CoV are highlighted in yellow and deep blue, respectively. Likewise, subunit 2 of SARS-CoV-2 and SARS-CoV are depicted in gray and forest green, respectively. **(B)** The sequence alignment of SARS-CoV-2 and SARS-CoV spike RBD. The yellow and gray shaded parts represent the RBD of both of the CoVs, and the RBM is represented in red. The conservancy and variation among subunit 1 (black boxes) and subunit 2 (pink boxes) are also shown in the alignment.

and 229E, share 35, 35, 37, 30, and 31% identity, respectively (**Supplementary Figures S3, S9**). Further, S protein mainly consists of receptor-binding domain (RBD) and receptor-binding motif (RBM), which are critically important for viral entry and attachment. The RBD of both SARS-CoV-2 and SARS-CoV shows high conservancy; however, it is important to notice that both of the subunits (S1 and S2) present in RBM show less conservancy, thus suggesting different modes and affinities to receptor binding and membrane fusion. The conservation and variation of RBD and RBM are shown in **Figures 4A,B**. The figure also depicts the receptor-binding S1 at amino-terminal and membrane fusion S2 subunits at carboxy-terminal along with RBD and RBM (**Figures 4A,B**). Further, some major structural differences between SARS-CoV and SARS-CoV-2 are depicted in **Figure 5**. Moreover, the active sites of S protein interacting with ACE2 are very critical for viral entry and transmission. We also analyzed and mapped the active sites, i.e., T402, R426, Y436, Y440, Y442, S460, L472, N473, Y475, N479, D480, Y484, T486, T487, G488, and Y491 of SARS-CoV S protein RBD on the SARS-CoV-2 S protein and marked the corresponding residues, which are structurally and sequentially conserved as putative active sites (**Figure 6**).

Based on the structural alignment, we found that amino acids at different positions, viz., T415, Y449, Y453, N487, Y489, T500, G502, and Y505, of SARS-CoV-2 S protein RBD remained the same, corresponding to the SARS-CoV S protein amino acids, i.e., T402, Y436, Y440, N473, Y475, T486, G488, and Y491, respectively (**Figure 6**). Furthermore, other amino acids, i.e., R and T at positions 426 and 487 of SARS-CoV was replaced by N at positions 439 and 501 of SARS-CoV-2, respectively. Likewise,

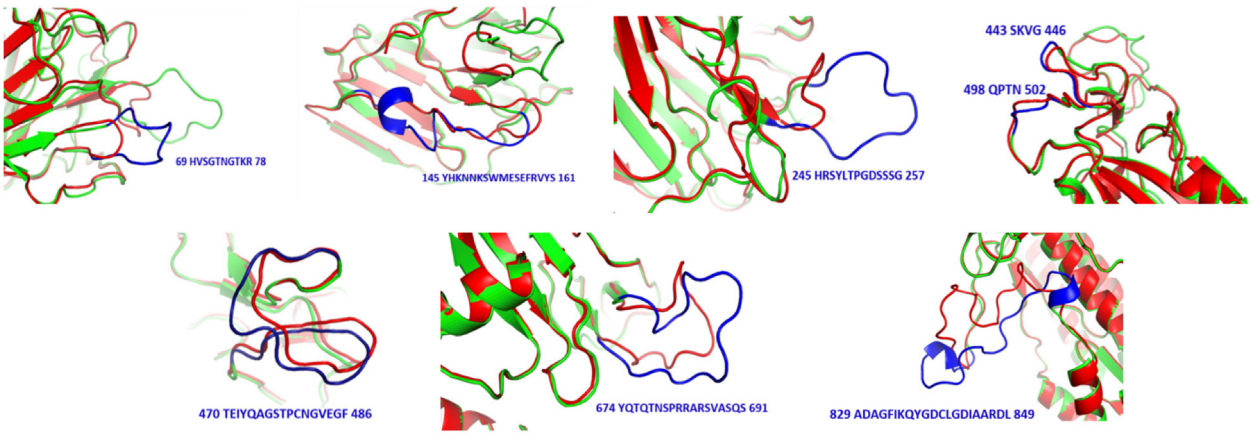


FIGURE 5 | Structural differences between SARS-CoV (red) and SARS-CoV-2 (green). Sequences and positions of SARS-CoV-2 regions are highlighted in blue.

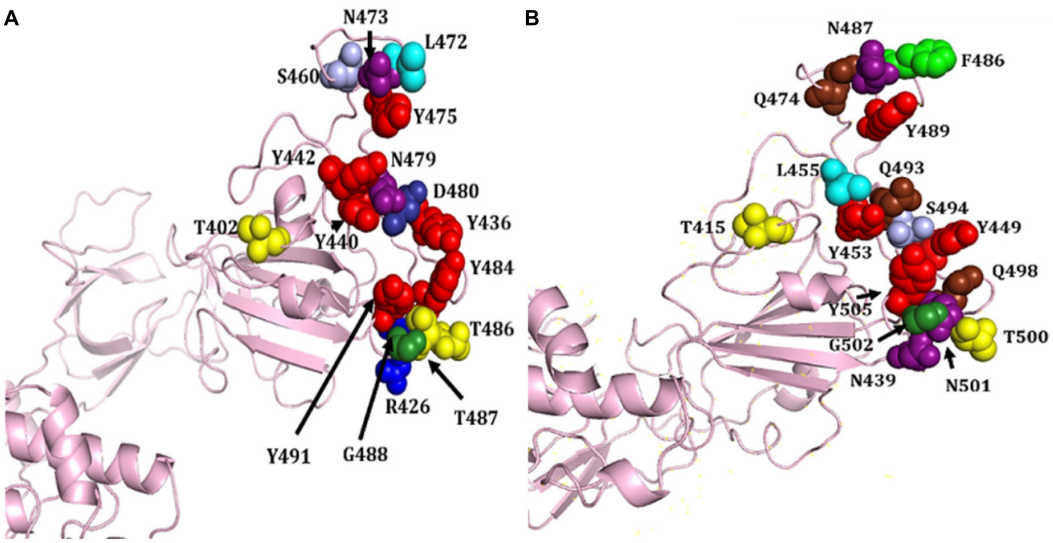


FIGURE 6 | Structural representation of various attachment sites of S protein RBD to ACE2. **(A)** Known active sites of SARS-CoV. **(B)** Mapped putative active sites on SARS-CoV-2 S protein corresponding to SARS-CoV S protein. Different amino acids are shown in distinct colors, i.e., R, T, N, Y, L, S, Q, F, G, D, and N.

L445 of SARS-CoV-2 replaced the aromatic amino acid Y442, Q at positions 474, 493, and 498 replaced S460, N479, and Y484 of SARS-CoV, respectively. The L472 and D480 of SARS-CoV were substituted by the aromatic amino acid F at positions 486 and S494 of SARS-CoV-2, respectively (**Figures 4B**, **6**).

As S protein may be an ideal target for vaccine design and development and, to date, there is no licensed vaccine or drug available for the treatment of the infection (COVID-19), a peptide vaccine could be designed based on S protein subunit 1, relying on the fact that ACE2 is the SARS-CoV-2 receptor (Shang et al., 2020). We have also depicted the predicted potential B cell (linear and discontinuous) (**Supplementary Figures S10, S11**) and T cell ($CD4^+$, $CD8^+$, and CTL) vaccine candidates on S protein (**Supplementary Figures S12, S13**). The four predicted efficient linear B-cell epitopes present at different locations are as follows: 369-YNSASFSTFKCYGVSPTKLNDLCFT-393 (25 AA), 404-GDEVRQIAPGQTGKIAD YNYKLP-426 (23 AA), 206-KHTPINLVRDLPQGFS-221 (17 AA), and 656-NNSYECDIPI-666 (11 AA) (**Supplementary Figure S10**), and three discontinuous epitopes are shown on trimeric S proteins (**Supplementary Figure S11**).

Further, predicted $CD4^+$ and $CD8^+$ epitopes of SARS-CoV-2 are depicted on the S protein (**Supplementary Figure S12**). The predicted epitopes are present at the 231-IGIN ITRFQTLLAH-245 (14 AA) and 61-NVTWFHAIHV-70 (10 AA) positions, respectively. Likewise, predicted CTL epitopes, i.e., 746-STECSNLLL-754, 821-LLFNKVTLA-829, 1053-VV FLHVTYV-1061, 827-TLADAGFIK-835, 507-PYRVVVLSF-515, 712-IAIPTNFTI-720, 886-WTFGAGAAL-894, 327-VRFPN ITNL-335, 505-YQPYRVVVL-513, 1016-AEIRASANL-1024, and 898-FAMQMAYRF-906 of length 9-mer, are also represented on the SARS-CoV-2 S protein (**Supplementary Figure S13**).

We have focused on structure prediction and conservation analysis of distinct proteins of seven different CoVs, including SARS-CoV-2. Comparisons between different coronavirus proteins provided valuable information on protein evolution, conservation, and variations to strategically develop antiviral agents against different CoVs, specifically for SARS-CoV-2. We also provide mapping of putative binding sites of S protein and potential epitopes for the active development of anti-SARS-CoV-2 agents. Moreover, high conservation against different proteins of SARS-CoV and SARS-CoV-2 provides an opportunity for the repurposing of small molecules and inhibitors and the development of cross-protective vaccine and antiviral therapy.

CONCLUSION

The ongoing infectious COVID-19 disease caused by SARS-CoV-2 has caused millions of deaths worldwide with no vaccine or therapeutic treatment to date to combat the deadly virus. To assist researchers in fighting SARS-CoV-2, we performed comprehensive meta-analyses and developed an integrative web-resource "CoronaVR." Largely, we focus on and recommend potential anti-SARS-CoV-2 solutions, i.e., T-cell and B-cell epitopes for incorporation into vaccine formulations, siRNA-based therapeutic regimens, and diagnostic primers. These can be useful candidates for researchers working toward developing anti-SARS-CoV-2 solutions.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

MKu conceived, designed, and supervised this study. AG performed the data collection and curation and developed the web server. MKh, AR, VS, PP, KB, and AG performed the vaccine epitope analysis. Sh, CS, and Ba performed analysis of diagnostic primers, siRNAs, and glycosylation sites. AT performed miRNA analysis. AM performed sgRNA analysis. SC and AM performed phylogenetic analysis. Sa performed codon analysis. PK and MKa performed the protein structure prediction and analysis. AG, MKh, and MKu performed the data interpretation and wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the Council of Scientific and Industrial Research (CSIR), Government of India (Grant Numbers OLP0501 and OLP0143). Funding for open access charge: Council of Scientific and Industrial Research (CSIR) (OLP0501 and OLP0143).

ACKNOWLEDGMENTS

We would like to acknowledge the Department of Biotechnology (DBT), Government of India (GAP0001), for infrastructural support.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.01858/full#supplementary-material

REFERENCES

Adedeji, A. O., and Lazarus, H. (2016). Biochemical characterization of middle east respiratory syndrome coronavirus helicase. *mSphere* 1, doi: 10.1128/mSphere.00235-16

Ahmed, F., and Raghava, G. P. (2011). Designing of highly effective complementary and mismatch siRNAs for silencing a gene. *PLoS One* 6:e23443. doi: 10.1371/journal.pone.0023443

Ahmed, S. F., Quadeer, A. A., and McKay, M. R. (2020). Preliminary Identification of Potential Vaccine Targets for the COVID-19 Coronavirus (SARS-CoV-2) Based on SARS-CoV Immunological Studies. *Viruses* 12:254. doi: 10.3390/v12030254

Baruah, V., and Bose, S. (2020). Immunoinformatics-aided identification of T cell and B cell epitopes in the surface glycoprotein of 2019-nCoV. *J. Med. Virol.* 92, 495–500. doi: 10.1002/jmv.25698

Benvenuto, D., Giovanetti, M., Ciccozzi, A., Spoto, S., Angeletti, S., and Ciccozzi, M. (2020a). The 2019-new coronavirus epidemic: evidence for virus evolution. *J. Med. Virol.* 92, 455–459. doi: 10.1002/jmv.25688

Benvenuto, D., Giovanetti, M., Salemi, M., Prosperi, M., De Flora, C., Junior Alcantara, L. C., et al. (2020b). The global spread of 2019-nCoV: a molecular evolutionary analysis. *Pathog. Glob. Health* 114, 64–67. doi: 10.1080/20477724.2020.1725339

Blom, N., Sicheritz-Ponten, T., Gupta, R., Gammeltoft, S., and Brunak, S. (2004). Prediction of post-translational glycosylation and phosphorylation of proteins from the amino acid sequence. *Proteomics* 4, 1633–1649. doi: 10.1002/pmic.200300771

Bogoch, I. I., Watts, A., Thomas-Bachli, A., Huber, C., Kraemer, M. U. G., and Khan, K. (2020). Potential for global spread of a novel coronavirus from China. *J. Travel Med.* 27:taaa011. doi: 10.1093/jtm/taaa011

Buels, R., Yao, E., Diesh, C. M., Hayes, R. D., Munoz-Torres, M., Helt, G., et al. (2016). JBrowse: a dynamic web platform for genome visualization and analysis. *Genome Biol.* 17:66. doi: 10.1186/s13059-016-0924-1

Bui, H. H., Sidney, J., Dinh, K., Southwood, S., Newman, M. J., and Sette, A. (2006). Predicting population coverage of T-cell epitope-based diagnostics and vaccines. *BMC Bioinformatics* 7:153. doi: 10.1186/1471-2105-7-153

Bui, H. H., Sidney, J., Li, W., Fusseder, N., and Sette, A. (2007). Development of an epitope conservancy analysis tool to facilitate the design of epitope-based diagnostics and vaccines. *BMC Bioinformatics* 8:361. doi: 10.1186/1471-2105-8-361

Calis, J. J., Maybeno, M., Greenbaum, J. A., Weiskopf, D., De Silva, A. D., Sette, A., et al. (2013). Properties of MHC class I presented peptides that enhance immunogenicity. *PLoS Comput. Biol.* 9:e1003266. doi: 10.1371/journal.pcbi.1003266

Casais, R., Dove, B., Cavanagh, D., and Britton, P. (2003). Recombinant avian infectious bronchitis virus expressing a heterologous spike gene demonstrates that the spike protein is a determinant of cell tropism. *J. Virol.* 77, 9084–9089. doi: 10.1128/jvi.77.16.9084-9089.2003

Castells, M., Victoria, M., Colina, R., Musto, H., and Cristina, J. (2017). Genome-wide analysis of codon usage bias in Bovine Coronavirus. *Virol. J.* 14:115. doi: 10.1186/s12985-017-0780-y

Chan, J. F., Lau, S. K., To, K. K., Cheng, V. C., Woo, P. C., and Yuen, K. Y. (2015). Middle East respiratory syndrome coronavirus: another zoonotic betacoronavirus causing SARS-like disease. *Clin. Microbiol. Rev.* 28, 465–522. doi: 10.1128/cmr.00102-14

Chang, C. K., Hou, M. H., Chang, C. F., Hsiao, C. D., and Huang, T. H. (2014). The SARS coronavirus nucleocapsid protein–forms and functions. *Antiviral Res.* 103, 39–50. doi: 10.1016/j.antiviral.2013.12.009

Chang, C. K., Sue, S. C., Yu, T. H., Hsieh, C. M., Tsai, C. K., Chiang, Y. C., et al. (2006). Modular organization of SARS coronavirus nucleocapsid protein. *J. Biomed. Sci.* 13, 59–72. doi: 10.1007/s11373-005-9035-9

Channappanavar, R., Zhao, J., and Perlman, S. (2014). T cell-mediated immune response to respiratory coronaviruses. *Immunol. Res.* 59, 118–128. doi: 10.1007/s12026-014-8534-z

Chen, J., Lau, Y. F., Lamirande, E. W., Paddock, C. D., Bartlett, J. H., Zaki, S. R., et al. (2010). Cellular immune responses to severe acute respiratory syndrome coronavirus (SARS-CoV) infection in senescent BALB/c mice: CD4+ T cells are important in control of SARS-CoV infection. *J. Virol.* 84, 1289–1301. doi: 10.1128/jvi.01281-09

Chen, N., Zhou, M., Dong, X., Qu, J., Gong, F., Han, Y., et al. (2020). Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study. *Lancet* 395, 507–513. doi: 10.1016/s0140-6736(20)30211-7

Chen, Y., Xu, Q., Yuan, X., Li, X., Zhu, T., Ma, Y., et al. (2017). Analysis of the codon usage pattern in Middle East Respiratory Syndrome Coronavirus. *Oncotarget* 8, 110337–110349. doi: 10.18632/oncotarget.22738

Cheng, Z. J., and Shan, J. (2020). 2019 Novel coronavirus: where we are and what we know. *Infection* 48, 155–163. doi: 10.1007/s15010-020-01401-y

Chesler, D. A., and Reiss, C. S. (2002). The role of IFN-gamma in immune responses to viral infections of the central nervous system. *Cytokine Growth Factor Rev.* 13, 441–454. doi: 10.1016/s1359-6101(02)00044-8

Colson, P., Rolain, J. M., and Raoult, D. (2020). Chloroquine for the 2019 novel coronavirus SARS-CoV-2. *Int. J. Antimicrob. Agents* 55:105923. doi: 10.1016/j.ijantimicag.2020.105923

de Haan, C. A., and Rottier, P. J. (2005). Molecular interactions in the assembly of coronaviruses. *Adv. Virus Res.* 64, 165–230. doi: 10.1016/s0065-3527(05)64006-7

de Wit, E., Feldmann, F., Cronin, J., Jordan, R., Okumura, A., Thomas, T., et al. (2020). Prophylactic and therapeutic remdesivir (GS-5734) treatment in the rhesus macaque model of MERS-CoV infection. *Proc. Natl. Acad. Sci. U.S.A.* 117:201922083. doi: 10.1073/pnas.1922083117

de Wit, E., van Doremalen, N., Falzarano, D., and Munster, V. J. (2016). SARS and MERS: recent insights into emerging coronaviruses. *Nat. Rev. Microbiol.* 14, 523–534. doi: 10.1038/nrmicro.2016.81

Del Rio, C., and Malani, P. N. (2020). 2019 novel coronavirus-important information for clinicians. *JAMA* doi: 10.1001/jama.2020.1490 [Epub ahead of print].

Dennis Lo, Y. M., and Chiu, R. W. K. (2020). Racing towards the development of diagnostics for a novel coronavirus (2019-nCoV). *Clin. Chem.* 66, 503–504. doi: 10.1093/clinchem/hvaa038

Dhanda, S. K., Karosiene, E., Edwards, L., Grifoni, A., Paul, S., Andreatta, M., et al. (2018). Predicting HLA CD4 immunogenicity in human populations. *Front. Immunol.* 9:1369. doi: 10.3389/fimmu.2018.01369

Dhanda, S. K., Vir, P., and Raghava, G. P. (2013). Designing of interferon-gamma inducing MHC class-II binders. *Biol. Direct.* 8:30. doi: 10.1186/1745-6150-8-30

Dimitrov, I., Bangov, I., Flower, D. R., and Doytchinova, I. (2014). AllerTOP v.2–a server for in silico prediction of allergens. *J. Mol. Model* 20:2278. doi: 10.1007/s00894-014-2278-5

Doytchinova, I. A., and Flower, D. R. (2007). VaxiJen: a server for prediction of protective antigens, tumour antigens and subunit vaccines. *BMC Bioinformatics* 8:4. doi: 10.1186/1471-2105-8-4

Du, L., Yang, Y., Zhou, Y., Lu, L., Li, F., and Jiang, S. (2017). MERS-CoV spike protein: a key target for antivirals. *Expert Opin. Ther. Targets* 21, 131–143. doi: 10.1080/14728222.2017.1271415

Du, L., Zhao, G., Chan, C. C., Li, L., He, Y., Zhou, Y., et al. (2010). A 219-mer CHO-expressing receptor-binding domain of SARS-CoV S protein induces potent immune responses and protective immunity. *Viral Immunol.* 23, 211–219. doi: 10.1089/vim.2009.0090

Du Toit, A. (2020). Outbreak of a novel coronavirus. *Nat. Rev. Microbiol.* 18:123. doi: 10.1038/s41579-020-0332-0

Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* 32, 1792–1797. doi: 10.1093/nar/gkh340

Fehr, A. R., and Perlman, S. (2015). Coronaviruses: an overview of their replication and pathogenesis. *Methods Mol. Biol.* 1282, 1–23. doi: 10.1007/978-1-4939-2438-7_1

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39, 783–791. doi: 10.1111/j.1558-5646.1985.tb00420.x

Gkirtzou, K., Tsamardinos, I., Tsakalides, P., and Poirazi, P. (2010). MatureBayes: a probabilistic algorithm for identifying the mature miRNA within novel precursors. *PLoS One* 5:e11843. doi: 10.1371/journal.pone.0011843

Goo, J., Jeong, Y., Park, Y. S., Yang, E., Jung, D. I., Rho, S., et al. (2020). Characterization of novel monoclonal antibodies against MERS-coronavirus spike protein. *Virus Res.* 278:197863. doi: 10.1016/j.virusres.2020.197863

Grifoni, A., Sidney, J., Zhang, Y., Scheuermann, R. H., Peters, B., and Sette, A. (2020). A Sequence Homology and Bioinformatic Approach Can Predict Candidate Targets for Immune Responses to SARS-CoV-2. *Cell Host Microbe* 27, 671-680.e2. doi: 10.1016/j.chom.2020.03.002

Guarner, J. (2020). Three emerging coronaviruses in two decades. *Am. J. Clin. Pathol.* 153, 420–421. doi: 10.1093/ajcp/aqaa029

Gupta, A. K., Kaur, K., Rajput, A., Dhanda, S. K., Sehgal, M., Khan, M. S., et al. (2016). ZikaVR: An Integrated Zika Virus Resource for Genomics, Proteomics, Phylogenetic and Therapeutic Analysis. *Sci. Rep.* 6:32713. doi: 10.1038/srep32713

Gupta, A. K., Kumar, A., Rajput, A., Kaur, K., Dar, S. A., Thakur, A., et al. (2020). NipahVR: a resource of multi-targeted putative therapeutics and epitopes for the Nipah virus. *Database* 2020:baz159. doi: 10.1093/database/baz159

Gupta, S., Kapoor, P., Chaudhary, K., Gautam, A., Kumar, R., and Raghava, G. P. (2013). In silico approach for predicting toxicity of peptides and proteins. *PLoS One* 8:e73957. doi: 10.1371/journal.pone.0073957

Hoffmann, M., Kleine-Weber, H., Schroeder, S., Kruger, N., Herrler, T., Erichsen, S., et al. (2020). SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor. *Cell* 181, 271-280.e8. doi: 10.1016/j.cell.2020.02.052

Hori, S., Nakazawa, H., Ohnishi, Y., Yoshino, H., Murayama, A., Nishikawa, Y., et al. (1989). A rapid cross-sectioning and freeze-clamping device for the beating canine heart. *J. Mol. Cell Cardiol.* 21, 203–210. doi: 10.1016/0022-2828(89)90862-6

Huang, C., Wang, Y., Li, X., Ren, L., Zhao, J., Hu, Y., et al. (2020). Clinical features of patients infected with 2019 novel coronavirus in Wuhan. *China. Lancet* 395, 497–506. doi: 10.1016/S0140-6736(20)30183-5

Hui, D. S., Esam, I. A., Madani, T. A., Ntoumi, F., Kock, R., Dar, O., et al. (2020). The continuing 2019-nCoV epidemic threat of novel coronaviruses to global health - The latest 2019 novel coronavirus outbreak in Wuhan, China. *Int. J. Infect Dis.* 91, 264–266. doi: 10.1016/j.ijid.2020.01.009

Hurst, K. R., Koetzner, C. A., and Masters, P. S. (2009). Identification of in vivo-interacting domains of the murine coronavirus nucleocapsid protein. *J. Virol.* 83, 7221–7234. doi: 10.1128/jvi.00440-09

Hurst, K. R., Koetzner, C. A., and Masters, P. S. (2013). Characterization of a critical interaction between the coronavirus nucleocapsid protein and nonstructural protein 3 of the viral replicase-transcriptase complex. *J. Virol.* 87, 9159–9172. doi: 10.1128/jvi.01275-13

Jespersen, M. C., Peters, B., Nielsen, M., and Marcatili, P. (2017). BepiPred-2.0: improving sequence-based B-cell epitope prediction using conformational epitopes. *Nucleic Acids Res.* 45, W24–W29. doi: 10.1093/nar/gkx346

Jiang, S., He, Y., and Liu, S. (2005). SARS vaccine development. *Emerg. Infect Dis.* 11, 1016–1020. doi: 10.3201/1107.050219

Julenius, K. (2007). NetCGlyc 1.0: prediction of mammalian C-mannosylation sites. *Glycobiology* 17, 868–876. doi: 10.1093/glycob/cwm050

Kaur, K., Gupta, A. K., Rajput, A., and Kumar, M. (2016). ge-CRISPR - An integrated pipeline for the prediction and analysis of sgRNAs genome editing efficiency for CRISPR/Cas system. *Sci. Rep.* 6:30870. doi: 10.1038/srep30870

Kickbusch, I., and Leung, G. (2020). Response to the emerging novel coronavirus outbreak. *BMJ* 368:m406. doi: 10.1136/bmj.m406

Kim, M. K., Yu, M. S., Park, H. R., Kim, K. B., Lee, C., Cho, S. Y., et al. (2011). 2,6-Bis-arylmethyloxy-5-hydroxychromones with antiviral activity against both hepatitis C virus (HCV) and SARS-associated coronavirus (SCV). *Eur. J. Med. Chem.* 46, 5698–5704. doi: 10.1016/j.ejmech.2011.09.005

Kim, Y., Ponomarenko, J., Zhu, Z., Tamang, D., Wang, P., Greenbaum, J., et al. (2012). Immune epitope database analysis resource. *Nucleic Acids Res.* 40, W525–W530. doi: 10.1093/nar/gks438

Ksiazek, T. G., Erdman, D., Goldsmith, C. S., Zaki, S. R., Peret, T., Emery, S., et al. (2003). A novel coronavirus associated with severe acute respiratory syndrome. *N. Engl. J. Med.* 348, 1953–1966. doi: 10.1056/NEJMoa030781

Kumar, S., Stecher, G., Li, M., Knyaz, C., and Tamura, K. (2018). MEGA X: molecular evolutionary genetics analysis across computing platforms. *Mol. Biol. Evol.* 35, 1547–1549. doi: 10.1093/molbev/msy096

Le, S. Q., and Gascuel, O. (2008). An improved general amino acid replacement matrix. *Mol. Biol. Evol.* 25, 1307–1320. doi: 10.1093/molbev/msn067

Liu, W. J., Zhao, M., Liu, K., Xu, K., Wong, G., Tan, W., et al. (2017). T-cell immunity of SARS-CoV: Implications for vaccine development against MERS-CoV. *Antiviral Res.* 137, 82–92. doi: 10.1016/j.antiviral.2016.11.006

Lu, H. (2020). Drug treatment options for the 2019-new coronavirus (2019-nCoV). *Biosci. Trends* 14, 69–71. doi: 10.5582/bst.2020.01020

Lu, L., Liu, Q., Zhu, Y., Chan, K. H., Qin, L., Li, Y., et al. (2014). Structure-based discovery of Middle East respiratory syndrome coronavirus fusion inhibitor. *Nat. Commun.* 5:3067. doi: 10.1038/ncomms4067

Lu, R., Zhao, X., Li, J., Niu, P., Yang, B., Wu, H., et al. (2020). Genomic characterisation and epidemiology of 2019 novel coronavirus: implications for virus origins and receptor binding. *Lancet* 395, 565–574. doi: 10.1016/s0140-6736(20)30251-8

Lucchese, G. (2020). Epitopes for a 2019-nCoV vaccine. *Cell Mol. Immunol.* 17, 539–540. doi: 10.1038/s41423-020-0377-z

Malik, Y. S., Sircar, S., Bhat, S., Sharun, K., Dhama, K., Dadar, M., et al. (2020). Emerging novel coronavirus (2019-nCoV)-current scenario, evolutionary perspective based on genome analysis and recent developments. *Vet. Q.* 40, 68–76. doi: 10.1080/01652176.2020.1727993

Maxmen, A. (2020). More than 80 clinical trials launch to test coronavirus treatments. *Nature* 578, 347–348. doi: 10.1038/d41586-020-00444-3

McKeever, T. M., Lewis, S. A., Smith, C., and Hubbard, R. (2004). Vaccination and allergic disease: a birth cohort study. *Am. J. Public Health* 94, 985–989. doi: 10.2105/ajph.94.6.985

Momattin, H., Al-Ali, A. Y., and Al-Tawfiq, J. A. (2019). A Systematic Review of therapeutic agents for the treatment of the Middle East Respiratory Syndrome Coronavirus (MERS-CoV). *Travel. Med. Infect. Dis.* 30, 9–18. doi: 10.1016/j.tmaid.2019.06.012

Morse, J. S., Lalonde, T., Xu, S., and Liu, W. R. (2020). Learning from the Past: Possible Urgent Prevention and Treatment Options for Severe Acute Respiratory Infections Caused by 2019-nCoV. *Chembiochem* 21, 730–738. doi: 10.1002/cbic.202000047

Moura, G., Pinheiro, M., Silva, R., Miranda, I., Afreixo, V., Dias, G., et al. (2005). Comparative context analysis of codon pairs on an ORFeome scale. *Genome Biol.* 6:R28. doi: 10.1186/gb-2005-6-3-r28

Nagpal, G., Chaudhary, K., Dhanda, S. K., and Raghava, G. P. S. (2017). Computational Prediction of the Immunomodulatory Potential of RNA Sequences. *Methods Mol. Biol.* 1632, 75–90. doi: 10.1007/978-1-4939-7138-1_5

Nielsen, M., Lundegaard, C., and Lund, O. (2007). Prediction of MHC class II binding affinity using SMM-align, a novel stabilization matrix alignment method. *BMC Bioinformatics* 8:238. doi: 10.1186/1471-2105-8-238

Perrier, A., Bonnin, A., Desmarets, L., Danneels, A., Goffard, A., Rouille, Y., et al. (2019). The C-terminal domain of the MERS coronavirus M protein contains a trans-Golgi network localization signal. *J. Biol. Chem.* 294, 14406–14421. doi: 10.1074/jbc.ra119.008964

Peters, B., and Sette, A. (2005). Generating quantitative models describing the sequence specificity of biological processes with the stabilized matrix method. *BMC Bioinformatics* 6:132. doi: 10.1186/1471-2105-6-132

Poh, C. M., Carissimo, G., Wang, B., Amrun, S. N., Lee, C. Y., Chee, R. S., et al. (2020). Two linear epitopes on the SARS-CoV-2 spike protein that elicit neutralising antibodies in COVID-19 patients. *Nat. Commun.* 11:2806. doi: 10.1038/s41467-020-16638-2

Ponomarenko, J., Bui, H. H., Li, W., Fusseder, N., Bourne, P. E., Sette, A., et al. (2008). ElliPro: a new structure-based tool for the prediction of antibody epitopes. *BMC Bioinformatics* 9:514. doi: 10.1186/1471-2105-9-514

Puigbo, P. I, Bravo, G., and Garcia-Vallve, S. (2008). CAIcal: a combined set of tools to assess codon usage adaptation. *Biol. Direct.* 3:38. doi: 10.1186/1745-6150-3-38

Qiu, T., Mao, T., Wang, Y., Zhou, M., Qiu, J., Wang, J., et al. (2020). Identification of potential cross-protective epitope between a new type of coronavirus (2019-nCoV) and severe acute respiratory syndrome virus. *J. Genet. Genomics* 47, 115–117. doi: 10.1016/j.jgg.2020.01.003

Qureshi, A., Thakur, N., and Kumar, M. (2013). VIRsiRNApred: a web server for predicting inhibition efficacy of siRNAs targeting human viruses. *J. Transl. Med.* 11:305. doi: 10.1186/1479-5876-11-305

Roper, R. L., and Rehm, K. E. (2009). SARS vaccines: where are we? *Expert. Rev. Vaccines* 8, 887–898. doi: 10.1586/erv.09.43

Ryu, S., and Chun, B. C. (2020). An interim review of the epidemiological characteristics of 2019 novel coronavirus. *Epidemiol. Health* 42:e2020006. doi: 10.4178/epih.e2020006

Sanchez-Trincado, J. L., Gomez-Perosanz, M., and Reche, P. A. (2017). Fundamentals and Methods for T- and B-Cell Epitope Prediction. *J. Immunol. Res.* 2017:2680160. doi: 10.1155/2017/2680160

Schoeman, D., and Fielding, B. C. (2019). Coronavirus envelope protein: current knowledge. *Virol. J.* 16:69. doi: 10.1186/s12985-019-1182-0

Shang, W., Yang, Y., Rao, Y., and Rao, X. (2020). The outbreak of SARS-CoV-2 pneumonia calls for viral vaccines. *NPJ Vaccines* 5:18. doi: 10.1038/s41541-020-0170-0

Sheahan, T. P., Sims, A. C., Leist, S. R., Schafer, A., Won, J., Brown, A. J., et al. (2020). Comparative therapeutic efficacy of remdesivir and combination lopinavir, ritonavir, and interferon beta against MERS-CoV. *Nat. Commun.* 11:222. doi: 10.1038/s41467-019-13940-6

Sheikh, A., Al-Taher, A., Al-Nazawi, M., Al-Mubarak, A. I., and Kandeel, M. (2020). Analysis of preferred codon usage in the coronavirus N genes and their implications for genome evolution and vaccine design. *J. Virol. Methods* 277:113806. doi: 10.1016/j.jviromet.2019.113806

Shen, L., Niu, J., Wang, C., Huang, B., Wang, W., Zhu, N., et al. (2019). High-Throughput Screening and Identification of Potent Broad-Spectrum Inhibitors of Coronaviruses. *J. Virol.* 93, doi: 10.1128/JVI.00023-19

Shi, J., Zhang, J., Li, S., Sun, J., Teng, Y., Wu, M., et al. (2015). Epitope-Based Vaccine Target Screening against Highly Pathogenic MERS-CoV: an In Silico Approach Applied to Emerging Infectious Diseases. *PLoS One* 10:e0144475. doi: 10.1371/journal.pone.0144475

Sidney, J., Steen, A., Moore, C., Ngo, S., Chung, J., Peters, B., et al. (2010). Divergent motifs but overlapping binding repertoires of six HLA-DQ molecules frequently expressed in the worldwide human population. *J. Immunol.* 185, 4189–4198. doi: 10.4049/jimmunol.1001006

Smith, T. R. F., Patel, A., Ramos, S., Elwood, D., Zhu, X., Yan, J., et al. (2020). Immunogenicity of a DNA vaccine candidate for COVID-19. *Nat. Commun.* 11:2601. doi: 10.1038/s41467-020-16505-0

Srivastava, S., Kamthania, M., Kumar Pandey, R., Kumar Saxena, A., Saxena, V., Kumar Singh, S., et al. (2019). Design of novel multi-epitope vaccines against severe acute respiratory syndrome validated through multistage molecular interaction and dynamics. *J. Biomol. Struct. Dyn.* 37, 4345–4360. doi: 10.1080/07391102.2018.1548977

Srivastava, S., Kamthania, M., Singh, S., Saxena, A. K., and Sharma, N. (2018). Structural basis of development of multi-epitope vaccine against Middle East respiratory syndrome using in silico approach. *Infect. Drug Resist.* 11, 2377–2391. doi: 10.2147/idr.s175114

Steentoft, C., Vakhrushev, S. Y., Joshi, H. J., Kong, Y., Vester-Christensen, M. B., Schjoldager, K. T., et al. (2013). Precision mapping of the human O-GalNAc glycoproteome through SimpleCell technology. *EMBO J.* 32, 1478–1488. doi: 10.1038/emboj.2013.79

Stranzl, T., Larsen, M. V., Lundegaard, C., and Nielsen, M. (2010). NetCTLpan: pan-specific MHC class I pathway epitope predictions. *Immunogenetics* 62, 357–368. doi: 10.1007/s00251-010-0441-4

Sturman, L. S., Holmes, K. V., and Behnke, J. (1980). Isolation of coronavirus envelope glycoproteins and interaction with the viral nucleocapsid. *J. Virol.* 33, 449–462. doi: 10.1128/jvi.33.1.449-462.1980

Sullivan, C. S., and Grundhoff, A. (2007). Identification of viral microRNAs. *Methods Enzymol.* 427, 3–23. doi: 10.1016/S0076-6879(07)27001-6

Tahir Ul Qamar, M., Saleem, S., Ashfaq, U. A., Bari, A., Anwar, F., and Alqahtani, S. (2019). Epitope-based peptide vaccine design and target site depiction against Middle East Respiratory Syndrome Coronavirus: an immune-informatics study. *J. Transl Med.* 17:362. doi: 10.1186/s12967-019-2116-8

Tanner, J. A., Zheng, B. J., Zhou, J., Watt, R. M., Jiang, J. Q., Wong, K. L., et al. (2005). The adamantane-derived bananins are potent inhibitors of the helicase activities and replication of SARS coronavirus. *Chem. Biol.* 12, 303–311. doi: 10.1016/j.chembiol.2005.01.006

The, L. (2020). Emerging understandings of 2019-nCoV. *Lancet* 395:311. doi: 10.1016/s0140-6736(20)30186-0

Tian, X., Li, C., Huang, A., Xia, S., Lu, S., Shi, Z., et al. (2020). Potent binding of 2019 novel coronavirus spike protein by a SARS coronavirus-specific human monoclonal antibody. *Emerg. Microbes Infect.* 9, 382–385. doi: 10.1080/22221751.2020.1729069

Totura, A. L., and Bavari, S. (2019). Broad-spectrum coronavirus antiviral drug discovery. *Expert. Opin. Drug. Discov.* 14, 397–412. doi: 10.1080/17460441.2019.1581171

Wang, C., Horby, P. W., Hayden, F. G., and Gao, G. F. (2020). A novel coronavirus outbreak of global health concern. *Lancet* 395, 470–473. doi: 10.1016/s0140-6736(20)30185-9

Wang, M., Cao, R., Zhang, L., Yang, X., Liu, J., Xu, M., et al. (2020). Remdesivir and chloroquine effectively inhibit the recently emerged novel coronavirus (2019-nCoV) in vitro. *Cell Res.* 30, 269–271. doi: 10.1038/s41422-020-0282-0

Wang, P., Sidney, J., Dow, C., Mothe, B., Sette, A., and Peters, B. (2008). A systematic assessment of MHC class II peptide binding predictions and evaluation of a consensus approach. *PLoS Comput. Biol.* 4:e1000048. doi: 10.1371/journal.pcbi.1000048

Wang, Y. D., Sin, W. Y., Xu, G. B., Yang, H. H., Wong, T. Y., Pang, X. W., et al. (2004). T-cell epitopes in severe acute respiratory syndrome (SARS) coronavirus spike protein elicit a specific T-cell immune response in patients who recover from SARS. *J. Virol.* 78, 5612–5618. doi: 10.1128/jvi.78.11.5612-5618.2004

Waterhouse, A., Bertoni, M., Bienert, S., Studer, G., Tauriello, G., Gumienny, R., et al. (2018). SWISS-MODEL: homology modelling of protein structures and complexes. *Nucleic Acids Res.* 46, W296–W303. doi: 10.1093/nar/gky427

Watts, C. H., Vallance, P., and Whitty, C. J. M. (2020). Coronavirus: global solutions to prevent a pandemic. *Nature* 578:363. doi: 10.1038/d41586-020-00457-y

Wrapp, D., Wang, N., Corbett, K. S., Goldsmith, J. A., Hsieh, C. L., Abiona, O., et al. (2020). Cryo-EM structure of the 2019-nCoV spike in the prefusion conformation. *Science* 367, 1260–1263. doi: 10.1126/science.abb2507

Wu, F., Zhao, S., Yu, B., Chen, Y. M., Wang, W., Song, Z. G., et al. (2020). A new coronavirus associated with human respiratory disease in China. *Nature* 579, 265–269. doi: 10.1038/s41586-020-2008-3

Xia, S., Yan, L., Xu, W., Agrawal, A. S., Algaissi, A., Tseng, C. K., et al. (2019). A pan-coronavirus fusion inhibitor targeting the HR1 domain of human coronavirus spike. *Sci. Adv.* 5:eaav4580. doi: 10.1126/sciadv.aav4580

Xu, X., and Gao, X. (2004). Immunological responses against SARS-coronavirus infection in humans. *Cell Mol. Immunol.* 1, 119–122.

Yi, C., Sun, X., Ye, J., Ding, L., Liu, M., Yang, Z., et al. (2020). Key residues of the receptor binding motif in the spike protein of SARS-CoV-2 that interact with ACE2 and neutralizing antibodies. *Cell Mol. Immunol.* 17, 621–630. doi: 10.1038/s41423-020-0458-z

Yong, C. Y., Ong, H. K., Yeap, S. K., Ho, K. L., and Tan, W. S. (2019). Recent advances in the vaccine development against middle east respiratory syndrome-coronavirus. *Front. Microbiol.* 10:1781. doi: 10.3389/fmicb.2019.01781

Yoon, H., and Leitner, T. (2015). PrimerDesign-M: a multiple-alignment based multiple-primer design tool for walking across variable genomes. *Bioinformatics* 31, 1472–1474. doi: 10.1093/bioinformatics/btu832

Yuan, M., Wu, N. C., Zhu, X., Lee, C. D., So, R. T. Y., Lv, H., et al. (2020). A highly conserved cryptic epitope in the receptor binding domains of SARS-CoV-2 and SARS-CoV. *Science* 368, 630–633. doi: 10.1126/science.abb7269

Zhang, H., Penninger, J. M., Li, Y., Zhong, N., and Slutsky, A. S. (2020). Angiotensin-converting enzyme 2 (ACE2) as a SARS-CoV-2 receptor: molecular mechanisms and potential therapeutic target. *Intensive Care Med.* 46, 586–590. doi: 10.1007/s00134-020-05985-9

Zhang, J., Zhou, L., Yang, Y., Peng, W., Wang, W., and Chen, X. (2020). Therapeutic and triage strategies for 2019 novel coronavirus disease in fever clinics. *Lancet Respir. Med.* 8, e11–e12. doi: 10.1016/s2213-2600(20)30071-0

Zhang, L., and Liu, Y. (2020). Potential interventions for novel coronavirus in China: a systematic review. *J. Med. Virol.* 92, 479–490. doi: 10.1002/jmv.25707

Zhao, S., Zhang, Q., Liu, X., Wang, X., Zhang, H., Wu, Y., et al. (2008). Analysis of synonymous codon usage in 11 human bocavirus isolates. *Biosystems* 92, 207–214. doi: 10.1016/j.biosystems.2008.01.006

Zhu, N., Zhang, D., Wang, W., Li, X., Yang, B., Song, J., et al. (2020). A Novel Coronavirus from Patients with Pneumonia in China, 2019. *N. Engl. J. Med.* 382, 727–733. doi: 10.1056/NEJMoa2001017

Zumla, A., Chan, J. F., Azhar, E. I., Hui, D. S., and Yuen, K. Y. (2016). Coronaviruses - drug discovery and therapeutic options. *Nat. Rev. Drug Discov.* 15, 327–347. doi: 10.1038/nrd.2015.37

Zumla, A., Memish, Z. A., Hui, D. S., and Perlman, S. (2019). Vaccine against Middle East respiratory syndrome coronavirus. *Lancet Infect. Dis.* 19, 1054–1055. doi: 10.1016/s1473-3099(19)30477-3

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Medicine

REVIEW
published: 31 July 2020
doi: 10.3389/fmed.2020.00480

Treatment Options for COVID-19: A Review

Mukarram Jamat Ali[1], *Muhammad Hanif*[2]*, *Muhammad Adnan Haider*[3], *Muhammad Umer Ahmed*[4], *FNU Sundas*[2], *Arham Hirani*[4], *Izhan Ali Khan*[5], *Khurram Anis*[6] *and Amin H. Karim*[7]

[1] Department of Medicine, King Edward Medical University, Lahore, Pakistan, [2] Department of Medicine, Khyber Medical College, Peshawar, Pakistan, [3] Department of Medicine, Allama Iqbal Medical College, Lahore, Pakistan, [4] Department of Medicine, Ziauddin University, Karachi, Pakistan, [5] Department of Medicine, Dow University of Health Sciences, Karachi, Pakistan, [6] Department of Gastroenterology, Pakistan Kidney and Liver Institute, Lahore, Pakistan, [7] Department of Cardiology, Baylor College of Medicine, Houston, TX, United States

Background: The recent COVID-19 pandemic sweeping the globe has caused great concern worldwide. Due to the limited evidence available on the dynamics of the virus and effective treatment options available, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has had a huge impact in terms of morbidity and mortality. The economic impact is still to be assessed.

Aims: The purpose of this article is to review the evidence for the multiple treatment options available, to consider the future of this global pandemic, and to identify some potential options that could revolutionize the treatment of COVID-19. Moreover, this article underscores the sheer importance of repurposing some of the available antiviral and antimicrobial agents that have long been in use so as to have an effective and expeditious response to this widespread pandemic and the need to conduct a multicenter global randomized controlled trial to find an effective single antiviral agent or a cocktail of available antimicrobial agents.

Method: We thoroughly searched and reviewed various case reports, retrospective analyses, and *in vitro* studies published in PubMed, EMBASE, and Google Scholar regarding the treatment options used for SARS-CoV, MERS-CoV, and SARS-CoV-2 since its outbreak in an attempt to highlight treatments with the most promising results.

Conclusion: We are currently facing one of the worst pandemics in history. Although SARS-CoV-2 is associated with a lower mortality rate than are SARS-CoV and MERS-CoV, its higher infectivity is making it a far more serious threat. Unfortunately, no vaccine against SARS-CoV-2 or effective drug regimen for COVID-19 currently exists. Drug repurposing of available antiviral agents may provide a respite; moreover, a cocktail of antiviral agents may be helpful in treating this disease. Here, we have highlighted a few available antimicrobial agents that could be very effective in treating COVID-19; indeed, a number of trials are underway to detect and confirm the efficacy of these agents.

Keywords: COVID-19, SARS-CoV-2, chloroquine/hydroxychloroquine, ivermectin, remdesivir, immunoglobulin, tocilizumab

OPEN ACCESS

Edited by:
Abdallah Samy,
University of Kansas, United States

Reviewed by:
Junki Maruyama,
University of Texas Medical Branch at Galveston, United States
Ishtiaq Hussain,
Cleveland Clinic Florida, United States
Koichi Yuki,
Boston Children's Hospital and Harvard Medical School, United States

***Correspondence:**
Muhammad Hanif
hanifafridi273@gmail.com

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: *02 May 2020*
Accepted: *15 July 2020*
Published: *31 July 2020*

Citation:
Ali MJ, Hanif M, Haider MA, Ahmed MU, Sundas F, Hirani A, Khan IA, Anis K and Karim AH (2020) Treatment Options for COVID-19: A Review. Front. Med. 7:480. doi: 10.3389/fmed.2020.00480

INTRODUCTION

In December 2019, the city of Wuhan, China, saw the outbreak of an unusual disease manifesting as severe pneumonia and respiratory distress. This disease epidemic was later shown to be caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and has now engulfed the world. The disease has spread across borders, leading to a global pandemic, and is currently showing no significant plateauing. SARS-CoV-2, formerly known as novel coronavirus (2019-nCOV), is a positive single-stranded RNA virus belonging to the family coronaviridae (1). Currently, there is not much strong evidence from randomized clinical trials to show improved outcomes or a decrease in terms of mortality with regards to the various treatment options available or prophylactic treatment. With little known about the virus and treatments available, we here highlight some of the leading therapeutic options and compare and contrast these in an attempt to determine which may be the most promising. We try to highlight up-to-date published clinical data and the treatment strategies for this novel pandemic so far.

Given the rapid spread so far and the new treatment modalities under consideration, the main focus lay on the repurposing of existing drugs, with several trials underway in attempts to find the most revolutionizing one.

TREATMENT MODALITIES COMPARED AND CONTRASTED

The treatment options available for COVID-19 and their mechanisms of action are briefly outlined in **Table 1**.

Chloroquine (CQ)/Hydroxychloroquine (HCQ)

CQ and HCQ have been used for the treatment of malaria for 70 years. CQ and HCQ act on multiple pathways of virus entry into and exit from cells and cause disruption of the essential viral protein synthesis (2). The *in-vitro* activities of CQ and HCQ have been shown to have an inhibitory effect on SARS-CoV-2 mRNA production, with HCQ showing greater efficacy than CQ (3, 4). However, *in vitro* activity cannot be interpreted as clinical activity against COVID-19; *in vitro* activity of CQ/HCQ against many other viruses, such as Ebola virus (5), Chikungunya virus (6), influenza virus (7), HIV (8), and dengue virus (9), has been reported previously, but their clinical efficacy did not reach that seen *in vitro*.

In a non-randomized trial in France on 36 patients with COVID-19, HCQ was administered alone or in combination with azithromycin. After 6 days of treatment, 100% of patients treated with the hydroxychloroquine and azithromycin combination had no detectable viral load in nasopharyngeal swabs compared to 57.1% of patients treated with hydroxychloroquine only and 12.5% of the control group ($p < 0.001$) (10). In another report from China in 100 patients with COVID-19, those treated with HCQ showed better clinical outcomes than control patients (11).

Triggered somewhat by the media and an intense pressure to prescribe a medication to COVID-19 patients and also due to the general perception of CQ/HCQ efficacy, clinicians may turn to off-label use of CQ/HCQ. Off-label use of CQ/HCQ is occurring globally, including in some hospitals in the USA but should be approached cautiously, as CQ and HCQ have a narrow therapeutic index and can cause QT interval prolongation, torsade de pointes, arrhythmia (12), bone marrow suppression, seizure, retinopathy, and myopathy.

Given the lack of evidence, we strongly call on public health organizations to collaborate effectively with local governments to support unified randomized controlled trials (RCTs) to test the potential therapeutic effects of CQ/HCQ against COVID-19. If the ethical use, safety, and advanced clinical efficacy of CQ/HCQ can be established by RCTs, as proposed by the WHO, it would be a significant advancement in the treatment of COVID-19 patients. Global multicenter RCTs would be the most effective approach for collecting accurate data about the safety and clinical efficacy of CQ/HCQ for the treatment of COVID-19, and this strategy would allow robust data to be available in the near future (13).

Azithromycin

Azithromycin is a bacteriostatic belonging to the macrolides class that inhibits bacterial protein synthesis and thus interferes with bacterial growth. It is also known to have antiviral effects in addition to its antibacterial properties. It has been used to treat respiratory viral infection due to this former property (14).

In a small non-randomized study conducted by Gautret et al., azithromycin in combination with HCQ has demonstrated substantial antiviral activity against SARS-CoV-2 (10). Literature on azithromycin alone as a treatment option for COVID-19 is scarce, and it is not clear whether macrolides can be used alone or should be in combination with HCQ. Masashi et al. believe that macrolides alone, or in combination with other drugs, are effective against SARS-CoV-2 (15).

Several clinical trials are being conducted to check the efficacy of HCQ-azithromycin for SARS-CoV-2. An interventional clinical trial is underway to determine the efficacy and safety of HCQ-azithromycin (16).

Remdesivir

Remdesivir is an adenosine analog that interferes with the synthesis of new viral RNA by chain termination. Although it was developed to be used against Ebola virus and Marburg virus infections (17), it showed antiviral activity against many other RNA viruses such as Lassa virus, respiratory syncytial virus, and coronaviruses such as SARS-CoV and MERS-CoV. Due to its antiviral activity against SARS-CoV and MERS-CoV, it has also been tested against SARS-CoV-2.

Remdesivir achieved satisfactory results in the Ces1c (–/–) mouse SARS model. It significantly reduced lung virus titers and improved pulmonary function when administered one day after disease onset ($p < 0.0001$). Virus titers were discernibly reduced, but with high mortality of mice, when administered 2 days after disease onset. This study concluded that when lung injury reaches a peak, simply reducing the virus titers can no longer suppress the strong immune responses in mice, but remdesivir

TABLE 1 | Different drugs available for COVID-19.

DRUGS	Mechanism of action	Adult dose/ administration drug	Contraindications	Toxicities	References
1-Choloroquine (CQ)/Hydroxy-Choloroquine (HCQ)	Interfere with viral entry & exit through cell and disruption of viral protein synthesis	Oral, HCQ 400 mg BID × 2 doses, then 400 mg q day × 4 days (five doses)	- Known hypersensitivity to CQ/HCQ and 4-aminoquinoline - Presence of retinal or visual field defects	- CQ/HCQ has a narrow therapeutic index - Can cause QT interval prolongation, torsade de pointes, arrhythmia - Bone marrow suppression - Seizure - Retinopathu - Myopathy	(2–13)
2- Azithromycin	Inhibits bacterial protein synthesis and also has some antiviral effect	Oral, 500 mg × 1, then 25 0 mg × 4 days (5 days total)	- Myasthenia Gravis - Hypokalemia - Hypomagnesemia - Tosade de pointes	- QT interval prolongation	(14–16)
3- Remdesivir	An adenosine analog; causes premature termination of the nascent viral RNA chains by incorporating into the viral genome	IV, 200 mg × 1, then 100 mg daily × 9 days (10 doses)		- Elevated level of transaminases - Kidney injury	(17–24)
4- Lopinavir/Ritonavir	An inhibitor of HIV type 1 protease (HIV-1); halts HIV-1 maturation and thus infectivity; the same for SARS-CoV-2	Oral, 400/100 mg BID × 10 days		**Common:** - GIT intolerance - Nausea/vomiting **Major:** - Pancreatitis - Hepatotoxicity - Cardiac conduction abnormalities	(25–36)
5- Favipiravir	Inhibits RNA-dependent RNA polymerase (RdRp) of RNA viruses which leads to chain termination	Oral, dosage varies Dosage adjustment requires in renal and liver diseases		- Neutropenia - Diarrhea - Hyperuricemia - Elevated level of transaminases	(37–43)
6- Ribavirin	A nucleoside analog of guanosine, inhibits RNA polymerase and acts as a chain terminator; is incorporated into the genome and causes mutations resulting in defective viral progeny - called "error catastrophe"	Oral, 400 mg TID (>50 ml/min), 400 mg BID (50–30 ml/min), 200 mg daily (<30 ml/min) × 10 days	- Pregnant women - Men whose female partners are pregnant - Patient with hemoglobinopathies	- Hemolytic Anemia - Tretogenic	(44–63)
7- Ivermectin	Anti-parasitic drug; has been shown to halt the replication of SARS-CoV-2 *in vitro*, as indicated by several-fold reduction of viral RNA			- Skin rash - Joint or muscle pain	(64–67)
8-Immunoglobulin	Antibodies obtained from recovered patients of COVID-19 can neutralize the virus when injected into new patients			- Flushing - Headache - Malaise - Fever - Renal impairment - Thrombosis - Arrhythmia	(68–76)
9- Corticosteroids	Corticosteroids play an anti -inflammatory role because of their various effects on various cytokines (1L-1, 1L-6, 1L-8, 1L-12, TNFα) and reduce pathological damage		- Patients with underlying infections - Diabetes - Hypertension	Short-term use does not cause any significant side effects, but long-term use can result in: - hypertension - diabetes - Osteoprosis - Weight gain	(77–81)

(Continued)

TABLE 1 | Continued

DRUGS	Mechanism of action	Adult dose/ administration drug	Contraindications	Toxicities	References
10. Interferon	Proteins secreted by cells of the immune system; boost the immune system			- Flu-like symptoms such as headache, fatigue, and weakness - Chills - Fever	(82–91)
11. Tocilizumab	Recombinant human IL-6 monoclonal antibody; binds to IL-6 receptors	IV, 400 mg (flat dose) × 1	- Patients with known hypersenstivity to tocilizumab - Caution in patients with neutropenia (<500 cells/micro L) or thrombocytopenia (<50,000 cells/micro L)	- Increase in upper respiratory tract infections like tuberculosis - Nasopharyngitis - Headache - Hypertension - Hematologic effects - Hepatotoxicity -GIT perforation - Hypersensitivity reactions	(92–102)

can significantly improve the symptoms and mortality ($p = 0.0037$) in mice when administered at early stages (18).

A case has been reported in which a patient with a SARS-CoV-2 infection confirmed by RT-PCR (performed on a nasopharyngeal swab) showed drastic improvement in one day with remdesivir (19). On account of the broad-spectrum anti-CoV activity of remdesivir, a randomized, double-blinded clinical trial was planned and is still ongoing (20). This study includes 308 participants, randomized to either remdesivir or placebo. Another phase 3 randomized, double-blinded, placebo-controlled study is underway focusing on the safety and efficacy of remdesivir in 452 hospitalized adults with severe respiratory symptoms from SARS-CoV-2 (21).

In an *in vitro* study, remdesivir inhibited the growth of bat-CoVs and human CoV (22). Another study revealed that remdesivir and chloroquine are very effective against SARS-CoV-2 *in vitro* (23).

Preliminary results from a recent randomized, placebo-controlled, double-blind phase 3 clinical trial in hospitalized patients with COVID-19 revealed that compared to placebo, remdesivir was associated with shorter time to recovery (11 vs. 15 days) (24)

Lopinavir/Ritonavir

Lopinavir is an inhibitor of HIV type 1 protease (HIV-1), halting the maturation of HIV-1 and thus its infectivity (25). Ritonavir, which is also a protease inhibitor, is administered in combination with lopinavir to enhance its bioavailability by inhibiting its metabolic inactivation (25). This combination is considered to be a highly effective antiretroviral agent, and some studies even advocate the use of monotherapy as a therapeutic option in certain HIV-infected patients (26). Along with other drugs (chloroquine, chlorpromazine, and loperamide), lopinavir was found to inhibit the *in vitro* replication of MERS-CoV and SARS-CoV (27). In patients with SARS associated with SARS-CoV infection, the combination of lopinavir/ritonavir and ribavirin resulted in a lower rate of acute respiratory distress syndrome (ARDS) or death at day 21 when compared to the historical control group treated with ribavirin only (2.4 vs. 28.8%, $p < 0.001$) (28). The lopinavir/ritonavir and ribavirin combination also allowed a reduction in steroid dosages and resulted in a decreased incidence of nosocomial infection (28). Lopinavir/ritonavir also reduced mortality in marmosets with MERS-like disease. The mortality rate at 36 h post-inoculation was 0–33% with lopinavir/ritonavir treatment vs. 67% in untreated or mycophenolate-treated animals (29). A case was reported in which a patient with MERS-CoV pneumonia improved and showed viral clearance after 6 days of triple antiviral therapy with lopinavir/ritonavir, ribavirin, and pegylated interferon (IFN)-alpha 2a (30). In another case, a patient with MERS-CoV pneumonia who later developed renal failure was started on triple antiviral therapy (lopinavir/ritonavir, ribavirin, and pegylated interferon) and showed resolution of viremia 2 days after treatment initiation, though virus shedding continued, highlighting the importance of starting ribavirin treatment early (31).

Given the effectiveness of lopinavir/ritonavir against MERS-CoV and SARS-CoV, it was thus tested for the treatment of SARS-CoV-2. Lopinavir, but not ritonavir, inhibits the *in vitro* replication of SARS-CoV-2 (32). Lopinavir/ritonavir was recommended for the treatment of SARS-CoV-2 pneumonia in China (33). A small report showed that out of four patients (two with mild SARS-CoV-2 pneumonia and two with severe) treated with lopinavir/ritonavir, umifenovir, and Shufeng Jiedu Capsule (a traditional Chinese medicine), three patients showed significant improvement and were discharged, while the other patient (with severe pneumonia) showed signs of improvement (34). In a patient with SARS-CoV-2 mild pneumonia, administration of lopinavir/ritonavir resulted in a decrease in the viral load from the very next day, and viral titers were later undetectable (35). The author highlighted that the decrease in viral titers could be due to the natural course of the disease; therefore, further studies are needed to determine the direct antiviral effect of lopinavir/ritonavir. Another young woman treated with lopinavir/ritonavir for

SARS-CoV-2 pneumonia showed improvement after 7 days of therapy (36).

Favipiravir

Favipiravir inhibits RNA-dependent RNA polymerase (RdRp) of RNA viruses (but not cellular RNA and DNA synthesis) (37) and shows broad-spectrum antiviral activity against RNA viruses (38). Favipiravir (T-705) can induce mutations in the genome of the influenza virus, which reduces the infectivity of the virus *in vitro*. This mechanism of lethal mutagenesis is proposed to be the key antiviral mechanism of favipiravir (39). It was originally developed against the influenza virus (38) and was the first effective drug against Ebola virus infection in an animal model (40). Favipiravir has shown *in vitro* effectiveness against the rabies virus (RABV) but is ineffective *in vivo*, especially after neuroinvasion. Although favipiravir blocked RABV replication at the site of inoculation in mice, it was not effective in the CNS, which means a method for its adequate penetration into the CNS needs to be devised (41). A randomized clinical trial in China comparing the efficacy of favipiravir and umifenovir for moderate symptoms showed that favipiravir is superior to umifenovir, having a higher recovery rate (71.4 vs. 55.9% for favipiravir and umifenovir, respectively; $p = 0.0199$). The time to cough relief and fever reduction by favipiravir was also shorter than that by umifenovir ($p < 0.0001$) (42). Further clinical trials of favipiravir in adult patients with SARS-CoV-2 pneumonia have been approved in China (43), and similar trials are being conducted at Harvard University and also in Japan.

Ribavirin

Ribavirin is a guanosine analog that acts as a chain terminator by inhibiting RNA polymerase (44). Alternative potential mechanisms could include its incorporation into the HCV genome, causing mutations and resulting in the production of defective viral progeny in a process called "error catastrophe" (45), or the inhibition of inosine monophosphate dehydrogenase (46). It is being used in combination with interferon in patients with chronic hepatitis C (47) and showed good results in patients with respiratory syncytial virus, especially immunocompromised patients (48).

The use of ribavirin in addition to corticosteroids in patients with SARS-CoV pneumonia resulted in resolution of fever and lung opacities within 2 weeks (49). In another study in Canada, ribavirin was administered to patients with clinical improvement, but no clear benefit was found. However, this study did highlight the side effects of ribavirin, as 49% of the patients showed a decrease in hemoglobin levels of 2 g/dL, and 76% showed signs of hemolysis, diagnosed by a 1.5 times increase in bilirubin or decreased haptoglobin level (50). In a series of 31 patients with SARS, 1 patient recovered with antibiotics only, 17 showed a rapid response to combination therapy (ribavirin and methylprednisolone), while the remaining required step-up or pulsed methylprednisolone therapy. This highlights the importance of ribavirin therapy in SARS (51). Ribavirin, when used in combination with interferon β, inhibits SARS-associated coronavirus replication *in vitro*; in another study, it showed antiviral activity against SARS coronavirus when used synergistically with interferon 1α and interferon β (52, 53). In a further study, it was able to lower the viral load in five out of eight patients (54). Ribavirin and interferon α2a given to MERS-CoV patients resulted in 14/20 (70%) survival as compared to 7/24 (29%) survival with no treatment at day 14 ($p = 0.04$) but 6/20 (30%) survival in the treatment group vs. 4/24 (17%) survival with no treatment at day 28 ($p = 0.54$). There was no significant difference between the later groups (55). It did not show any advantages in SARS-CoV patients (56, 57). Ribavirin used with interferon α in MERS-CoV resulted in improvement in 4 days in one patient and 6 days in another (58). No treatment advantage was seen with ribavirin in MERS-CoV after meta-analysis (59, 60). Ribavirin showed *in vitro* antiviral effects against SARS-CoV-2 (24). Ribavirin can be used for COVID-19 (61), and it has also been recommended to use for COVID-19 via intravenous infusion (62), as it binds tightly with SARS-CoV-2 RdRp and stops polymerase function (63). However, we need a randomized control trial to elucidate the antiviral potential of ribavirin. Moreover, it should be used in combination with either interferon or lopinavir/ritonavir to enhance its antiviral activity against SARS-CoV-2.

Ivermectin

This FDA-approved anti-parasitic drug has been shown to halt the replication of SARS-CoV-2 *in vitro*, as indicated by a several-fold reduction in viral RNA ivermectin-treated samples (64). However, further evaluation is needed to determine its efficacy in combating COVID-19 in humans. Ivermectin also shows broad-spectrum antiviral activity. It inhibits yellow fever virus replication, specifically targeting NS3 helicase activity (65). Ivermectin also inhibits HIV-1 (66) and Dengue virus (66, 67) replication by inhibiting importin alpha/beta, which facilitates the transport of proteins between the cytoplasm and nucleus, as these viruses use these proteins for their replication.

Immunoglobulin

IgG antibodies have two functional parts: Fab fragments, which help in antigen recognition, and the Fc fragment, which helps in the activation of the immune system (68). Intravenous immunoglobulin (IVIG) is effectively used for autoimmune diseases and chronic inflammatory diseases such as lupus, multiple sclerosis, Kawasaki disease, and dermatomyositis (69, 70). It has been used for the treatment of various bacterial, viral, and fungal infections in humans and in many experimental models (71, 72).

Likewise, SARS-Cov-2 infections could be treated using polyclonal antibodies from recovered COVID-19 patients (73). It would be preferable to extract the immunoglobulin from patients in the same city or the same area, as lifestyle, diet, and the environment are implicated in the development of specific antibodies against the virus. Immune IgG collected in China may be different from that collected in Europe or the USA (74).

In an uncontrolled case series, five critically ill COVID-19 patients on ventilators and receiving methylprednisolone and antiviral agents were transfused with convalescent plasma containing SARS-CoV-2 specific antibody (IgG) at a binding titer

>1:1,000 that had been obtained from five recovered COVID-19 patients. Convalescent plasma was transfused between days 10–22 after admission. Out of five patients, three were weaned from the ventilator after 2 weeks, and four out of five recovered from ARDS after 12 days of transfusion of convalescent plasma. Three patients were discharged after 51-, 53-, and 55-days stays at hospital, and the remaining two were in stable condition after 37 days of convalescent plasma transfusion (75). There were a few limitations to the above study. Firstly, this was a small case series with no control patients, and secondly, these patients had already been given antiviral agents and steroids.

This method of passive antibody therapy can provide an effective treatment against the rapidly rising pandemic of COVID-19 (76). Though serum antibodies have been in use as a treatment for a relatively long time, further clinical trials with control groups are needed to support the idea of using serum antibodies as a treatment option for COVID-19.

Corticosteroids

Corticosteroids are a class of steroid hormones that play a key physiological role in inflammation and the immune system. The use of corticosteroids for COVID-19 has been controversial since the outbreak of this disease (77). In the past, corticosteroids have been widely used for treatment during SARS-CoV outbreaks because of their effects on various cytokines [IL-1, IL-6, IL-8, IL-12, and tumor necrosis factor-α (TNF-α)] (78, 79). Studies in humans have shown that corticosteroids are effective in reducing pathological damage, but the main concern is their adverse effects, such as acute respiratory syndrome (79).

A study was conducted on the treatment of porcine respiratory coronavirus with dexamethasone and showed that one or two doses at earlier stages are effective in reducing pro-inflammatory responses but prolonged use may play a role in enhancing viral replication (80). Another Chinese study was conducted in which SARS-CoV patients were divided into four groups; this showed that early and high doses of steroids with quinolone had an effective response (56).

A randomized clinical trial will be conducted to determine the effectiveness of systemic glucocorticoids in patients with severe novel coronavirus pneumonia (6). The use of corticosteroids for COVID-19 is controversial because of the risks of acute respiratory syndrome and further enhancement of viral replication (81).

Interferon

Interferons are naturally occurring proteins produced and secreted by cells of the immune system, e.g., white blood cells, epithelial cells, and fibroblasts. There are three major classes of interferon (alpha, beta, and gamma). Each class has a different and diverse action. Interferons boost the immune system against invading antigens, such as viruses and bacteria, and affect not only the stimulated cell but also neighboring cells (82).

Literature reviews highlight that interferons have been in use for many years against emerging viruses when no other treatment options have been available (83). Interferons have also been used for SARS-CoV and MERS-CoV in the past and have shown promising results both *in vitro* and *in vivo* in decreasing viral replication (83–86). Most of the time, interferons were used in combination with ribavirin or lopinavir/ritonavir, but the potential benefits did not meet expectations, most probably because of their administration at later, post-infectious, stages (59).

From previous studies, we can assume that interferons may be an effective treatment option against SARS-CoV-2 (87). SARS-CoV and MERS-CoV are able to disrupt interferon signaling pathways by interfering with proteins involved in interferon expression, such as Orf6 and Orf3b (88). The excessive *in vitro* sensitivity of SARS-CoV-2 to interferons is potentially because SARS-CoV-2 might have lost these anti-interferon actions due to their truncated Orf6 and Orf3b proteins (89). This suggests that interferons may be a better potential treatment option for SARS-CoV-2 than for SARS-CoV. As interferon treatment is more effective at earlier stages, they can be used prophylactically against SARS-CoV-2, and this is further supported by the *in vitro* efficacy of interferon pretreatment against the virus (89). Shen et al. stated that interferon-2α can effectively reduce the infection rate of SARS-CoV-2, which further supports the above hypothesis (90).

The recommended guidelines for the treatment of SARS-CoV-2 in China include administering 5M units of interferon α via an inhaler in combination with oral ribavirin twice a day (62). The advantage of inhalation therapy is that it acts directly on the respiratory tract; however, the pharmacokinetics and pharmacodynamics of this route are not precisely known (87). A clinical trial is underway to determine the effectiveness of interferon α with ribavirin and lopinavir/ritonavir in COVID-19 patients (91).

Due to the greater sensitivity of SARS-CoV-2 to interferon α in comparison to its family members (SARS-CoV and MERS-CoV), it can be used as an effective treatment option for COVID-19 patients. However, it will be necessary to wait for the results from current clinical trials to understand the exact efficacy of interferon (87).

Tocilizumab

Tocilizumab is a recombinant anti-human interleukin (IL)-6 receptor monoclonal antibody. It binds to both membrane-bound and soluble IL-6 receptors (IL-6R) and prevents further inflammatory cascades (92). It has been seen that critical SARS-CoV-2 patients have a surge of inflammatory cytokines, called a cytokine storm, as was previously seen with SARS and MERS. These inflammatory markers (IL-1B, IL-1RA, IL-7, IL-8, IL-9, IL-10, fibroblast growth factor, granulocyte-macrophage colony stimulating factor, IFNγ, granulocyte-colony stimulating factor, interferon-γ-inducible protein, monocyte-derived growth factor, TNFα, and vascular endothelial growth factor) were high in COVID-19 patients, leading to systemic inflammation and multi-organ failure (93, 94). IL-6 and IL-2 receptor (IL-2R) can be used to predict the severity of COVID-19-related pneumonia, as significant differences between the levels of IL-6 and IL-2R were seen between the three clinically differentiated groups ($p < 0.05$). The study showed that the more severe the disease, the higher the levels of IL-6 and IL-2R (95). Several other reports have shown elevated levels of IL-6 in

COVID-19 critical patients (96). IL-6 is a key substance in cytokine release syndrome, so blocking IL-6R with tocilizumab can save patients with severe COVID-19 (97). In a small clinical trial in China, 21 patients with severe or critical COVID-19 were treated with tocilizumab. Within a few days of treatment, fever resolved in all patients, 15/20 (75%) needed less oxygen (one needed no oxygen), and CT scans showed resolution of pulmonary lesions in 19/21 (90.5%) patients. Lymphocytes in 10/19 (52.6%) patients and C-reactive protein (CRP) in 16/19 (84.2%) patients also returned to normal (98). In another retrospective study, tocilizumab was administered to 15 patients, and a significant improvement was seen in CRP levels, which dropped from 126.9 (10.7–257.9) to 11.2 (0.02–113.7) mg/L ($p < 0.01$) (99). Many individual cases have been reported in which the use of tocilizumab resulted in a significant improvement in patients. A 60-year-old patient with a previous history of multiple myeloma presented with chest tightness, and his chest CT showed multiple ground-glass opacities. He was admitted and given moxifloxacin for 3 days. Later, he was given umifenovir (arbidol), as the diagnosis of COVID-19 was confirmed by real time RT-PCR performed on a nasopharyngeal swab.

Two weeks later, he was transferred to another hospital as his chest tightness had worsened, and his oxygen saturation had become low. A CT scan showed bilateral, multiple ground-glass opacities. He was given methylprednisolone on days 2–6 of admission to improve his chest tightness and dyspnea. The patient still had bilateral, multiple ground-glass opacities on a chest CT performed on day 8. His laboratory results showed a high level of serum IL-6, and he was administered 8 mg/kg of IV tocilizumab. His IL-6 level started decreasing, chest tightness improved, and his CT on day 19 showed a decrease in ground-glass opacities (100).

Another 42-year-old patient with a history of renal cell carcinoma presented with fever and received ceftriaxone. After 6 days, he developed cough and fever, and his real-time PCR results for SARS-CoV-2 were positive. A further CT scan showed bilateral ground-glass opacities, and he was started on lopinavir/ritonavir (for 5 days) on day 7. On day 8, he developed dyspnea with decreasing oxygen saturation and was put on oxygen supplementation. He was given two doses of tocilizumab, 8 h apart, and his condition started improving. On day 12, partial regression of pulmonary infiltrates and ground-glass opacities was seen on chest CT. His CRP (a marker of cytokine storm) decreased from 225 mg/L to 33 mg/L in 4 days (101).

A 45-year-old male patient with a history of sickle cell disease presented with vaso-occlusive crises, no pulmonary findings, no dyspnea, no cough, no fever, and oxygen saturation of 98%. On day 1, the patient developed fever, and his oxygen saturation dropped to 91% with auscultatory crepitations. He was given amoxicillin-clavulanic acid and hydroxychloroquine, while a specimen was sent for RT-PCR testing for SARS-CoV-2. On day 3, his saturation dropped to 80% and his chest CT showed abnormal findings consistent with SARS-CoV-2-related pneumonia and acute chest syndrome. After RT-PCR results indicated SARS-CoV-2, a single dose of tocilizumab was injected and the patient improved and was discharged after blood transfusion (his hemoglobin was low) (102).

These cases highlight that tocilizumab can be used to successfully treat COVID-19 patients with respiratory failure by limiting cytokine-related pulmonary damage.

VACCINES

Vaccination can be the only definitive and preventive treatment option for COVID-19. A number of vaccine clinical trials are being conducted. A clinical trial by the University of Oxford is currently in phase 2/3 (103), and another phase 2 trial by the Institute of Biotechnology, Academy of Military Medical Sciences, PLA of China, is in progress. (104).

CONCLUSION

After reviewing a number of studies and case reports, we conclude that remdesivir and hydroxychloroquine/chloroquine with or without azithromycin are promising treatment options for patients with mild and moderate COVID-19. However, tocilizumab and immunoglobulin therapy seem to be effective in treating severe disease. There is a need for randomized control trials involving the entire globe to determine the efficacy and potency of these available potential treatment options.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

REFERENCES

1. Ahmed SF, Quadeer AA, McKay MR. Preliminary identification of potential vaccine targets for the COVID-19 coronavirus (SARS-CoV-2) based on SARS-CoV immunological studies. *Viruses*. (2020) 12:254. doi: 10.3390/v12030254
2. Fantini J, Di Scala C, Chahinian H, Yahi N. Structural and molecular modelling studies reveal a new mechanism of action of chloroquine and hydroxychloroquine against SARS-CoV-2 infection. *Int J Antimicrob Agents*. (2020) 55:105960. doi: 10.1016/j.ijantimicag.2020.105960
3. Yao X, Ye F, Zhang M, Cui C, Huang B, Niu P, et al. *In vitro* antiviral activity and projection of optimized dosing design of hydroxychloroquine for the treatment of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). *Clin Infect Dis*. (2020) 9:ciaa237. doi: 10.1093/cid/ciaa237. [Epub ahead of print].
4. Weston S, Haupt R, Logue J, Matthews K, Frieman M. FDA approved drugs with broad anti-coronaviral activity inhibit SARS-CoV-2 *in vitro*. *BioRxiv*. (2020). doi: 10.1101/2020.03.25.008482
5. Dowall SD, Bosworth A, Watson R, Bewley K, Taylor I, Rayner E, et al. Chloroquine inhibited Ebola virus replication in vitro but failed to protect against infection and disease in the *in vivo* guinea pig model. *J Gen Virol*. (2015) 96:3484–92. doi: 10.1099/jgv.0.000309
6. de Lamballerie X, Boisson V, Reynier JC, Enault S, Charrel RN, Flahault A, et al. On chikungunya acute infection and chloroquine treatment. *Vector Borne Zoonotic Dis*. (2008) 8:837–9. doi: 10.1089/vbz.2008.0049

7. Paton NI, Lee L, Xu Y, Ooi EE, Cheung YB, Archuleta S, et al. Chloroquine for influenza prevention: a randomised, double-blind, placebo controlled trial. *Lancet Infect Dis*. (2011) 11:677–83. doi: 10.1016/S1473-3099(11)70065-2
8. Sperber K, Louie M, Kraus T, Proner J, Sapira E, Lin S, et al. Hydroxychloroquine treatment of patients with human immunodeficiency virus type 1. *Clin Ther*. (1995) 17:622–36. doi: 10.1016/0149-2918(95)80039-5
9. Tricou V, Minh NN, van TP, Lee SJ, Farrar J, Wills B, et al. A randomized controlled trial of chloroquine for the treatment of dengue in Vietnamese adults. *PLoS Negl Trop Dis*. (2012) 4:e785. doi: 10.1371/annotation/8683caec-b309-46d7-bc47-dc9cc27108e4
10. Gautret P, Lagier JC, Parola P, Hoang VT, Meddeb L, Mailhe M, et al. Hydroxychloroquine and azithromycin as a treatment of COVID-19: results of an open-label non-randomized clinical trial. *Int J Antimicrob Agents*. (2020) 56:105949. doi: 10.1016/j.ijantimicag.2020.105949
11. Gao J, Tian Z, Yang X. Breakthrough: chloroquine phosphate has shown apparent efficacy in treatment of COVID-19 associated pneumonia in clinical studies. *Biosci Trends*. (2020) 14:72–3. doi: 10.5582/bst.2020.01047
12. Nord JE, Shah PK, Rinaldi RZ, Weisman MH. Hydroxychloroquine cardiotoxicity in systemic lupus erythematosus: a report of 2 cases and review of the literature. *Semin Arthritis Rheum*. (2004) 33:336–51. doi: 10.1016/j.semarthrit.2003.09.012
13. ClinicalTrials.gov Schilling W. *National Library of Medicine (U.S). (2020). March, 11-. Identifier: NCT04303507 chloroquine/Hydroxychloroquine Prevention of Coronavirus Disease (COVID-19) in the Healthcare Setting (COPCOV)*. Available online at: https://clinicaltrials.gov/ct2/show/NCT04303507 (accessed Jun 24, 2020).
14. Min JY, Jang YJ. Macrolide therapy in respiratory viral infections. *Mediators Inflamm*. (2012) 2012:649570. doi: 10.1155/2012/649570
15. Ohe M, Shida H, Jodo S, Kusunoki Y, Seki M, Furuya K, et al. Macrolide treatment for COVID-19: Will this be the way forward? *Biosci Trends*. (2020) 14:159–60. doi: 10.5582/bst.2020.03058
16. ClinicalTrials.gov. *National Library of Medicine (U.S). (2020). March, 25-. Identifier: NCT04321278 Safety and Efficacy of hydroxychloroquine associated with azithromycin in SARS-CoV-2 virus (coalition Covid-19 Brasil II)*. Available online at: https://clinicaltrials.gov/ct2/show/NCT04321278 (accessed Jun 24, 2020).
17. Warren TK, Jordan R, Lo MK, Ray AS, Mackman RL, Soloveva V, et al. Therapeutic efficacy of the small molecule GS-5734 against Ebola virus in rhesus monkeys. *Nature*. (2016) 531:381–5. doi: 10.1038/nature17180
18. Sheahan TP, Sims AC, Leist SR, Schäfer A, Won J, Brown AJ, et al. Comparative therapeutic efficacy of remdesivir and combination lopinavir, ritonavir, and interferon beta against MERS-CoV. *Nat Commun*. (2020) 11:222. doi: 10.1038/s41467-019-13940-6
19. Holshue ML, deBolt C, Lindquist S, Lofy KH, Wiesman J, Bruc H, et al. First case of 2019 novel coronavirus in the United States. *N Engl J Med*. (2020) 382:929–36. doi: 10.1056/NEJMoa2001191
20. ClinicalTrials.gov. *National Library of Medicine (U.S)*. (2020). *Feb, 05-. Identifier: NCT04252664 A Trial of Remdesivir in Adults with Mild and Moderate COVID-19*. Available online at: https://clinicaltrials.gov/ct2/show/results/NCT04252664 (accessed June 24, 2020).
21. ClinicalTrials.gov. *National Library of Medicine (U.S)*. (2020). *Feb, 06-. Identifier: NCT04257656 A Trial of Remdesivir in Adults with severe COVID-19*. Available from: https://clinicaltrials.gov/ct2/show/NCT04257656 (accessed June 24, 2020).
22. Agostini ML, Andres EL, Sims AC, Graham RL, Sheahan TP, Lu X, et al. Coronavirus susceptibility to the antiviral Remdesivir (GS-5734) is mediated by the viral polymerase and the proofreading exoribonuclease. *mBio*. (2018) 9:e00221-18. doi: 10.1128/mBio.00221-18
23. Wang M, Cao R, Zhang L, Yang X, Liu J, Xu M, et al. Remdesivir and chloroquine effectively inhibit the recently emerged novel coronavirus (2019-nCoV) *in vitro*. *Cell Res*. (2020) 30:269–71. doi: 10.1038/s41422-020-0282-0
24. Beigel JH, Tomashek KM, Dodd LE, Mehta AK, Zingman BS, Kalil AC, et al. Remdesivir for the treatment of covid-19 - preliminary report. *N Engl J Med*. (2020) NEJMoa2007764. doi: 10.1056/NEJMoa2007764
25. Cvetkovic RS, Goa KL. Lopinavir/ritonavir: a review of its use in the management of HIV infection. *Drugs*. (2003) 63:769–802. doi: 10.2165/00003495-200363080-00004
26. Chandwani A, Shuter J. Lopinavir/ritonavir in the treatment of HIV-1 infection: a review. *Ther Clin Risk Manag*. (2008) 4:1023–33. doi: 10.2147/TCRM.S3285
27. de Wilde AH, Jochmans D, Posthuma CC, Zevenhoven-Dobbe JC, van Nieuwkoop S, Bestebroer TM, et al. Screening of an FDA-approved compound library identifies four small-molecule inhibitors of Middle East respiratory syndrome coronavirus replication in cell culture. *Antimicrob Agents Chemother*. (2014) 58:4875–84. doi: 10.1128/AAC.03011-14
28. Chu CM, Cheng VC, Hung IF, Wong MM, Chan KH, Chan KS, et al. Role of lopinavir/ritonavir in the treatment of SARS: initial virological and clinical findings. *Thorax*. (2004) 59:252–6. doi: 10.1136/thorax.2003.012658
29. Chan JF, Yao Y, Yeung ML, Deng W, Bao L, Jia L, et al. Treatment with lopinavir/ritonavir or interferon-β1b improves outcome of MERS-CoV infection in a nonhuman primate model of common marmoset. *J Infect Dis*. (2015) 212:1904–13. doi: 10.1093/infdis/jiv392
30. Kim UJ, Won EJ, Kee SJ, Jung SI, Jang HC. Combination therapy with lopinavir/ritonavir, ribavirin and interferon-α for middle East respiratory syndrome. *Antivir Ther*. (2016) 21:455–9. doi: 10.3851/IMP3002
31. Spanakis N, Tsiodras S, Haagmans BL, Raj VS, Pontikis K, Koutsoukou A, et al. Virological and serological analysis of a recent Middle East respiratory syndrome coronavirus infection case on a triple combination antiviral regimen. *Int J Antimicrob Agents*. (2014) 44:528–32. doi: 10.1016/j.ijantimicag.2014.07.026
32. Choy KT, Wong AY, Kaewpreedee P, Sia SF, Chen D, Hui KPY, et al. Remdesivir, lopinavir, emetine, and homoharringtonine inhibit SARS-CoV-2 replication *in vitro*. *Antiviral Res*. (2020) 178:104786. doi: 10.1016/j.antiviral.2020.104786
33. Jin YH, Cai L, Cheng ZS, Cheng H, Deng T, Fan Y-P, et al. A rapid advice guideline for the diagnosis and treatment of 2019 novel coronavirus (2019-nCoV) infected pneumonia (standard version). *Mil Med Res*. (2020) 7:4. doi: 10.1186/s40779-020-0233-6
34. Wang Z, Chen X, Lu Y, Chen F, Zhang W. Clinical characteristics and therapeutic procedure for four cases with 2019 novel coronavirus pneumonia receiving combined Chinese and Western medicine treatment. *Biosci Trends*. (2020) 14:64–8. doi: 10.5582/bst.2020.01030
35. Lim J, Jeon S, Shin HY, Kim MJ, Seong YM, Lee WJ, et al. Case of the index patient who caused tertiary transmission of COVID-19 infection in Korea: the application of lopinavir/ritonavir for the treatment of COVID-19 infected pneumonia monitored by quantitative RT-PCR. *J Korean Med Sci*. (2020) 35:e79. doi: 10.3346/jkms.2020.35.e79
36. Kim JY, Choe PG, Oh Y, Kim J, Park SJ, Park JH, et al. The first case of 2019. novel coronavirus pneumonia imported into Korea from Wuhan, China: implication for infection prevention and control measures. *J Korean Med Sci*. (2020) 35:e61. doi: 10.3346/jkms.2020.35.e61
37. Furuta Y, Takahashi K, Kuno-Maekawa M, Sangawa H, Uehara S, Kozaki K, et al. Mechanism of action of T-705 against influenza virus. *Antimicrob Agents Chemother*. (2005) 49:981–6. doi: 10.1128/AAC.49.3.981-986.2005
38. Furuta Y, Komeno T, Nakamura T. Favipiravir (T-705), a broad spectrum inhibitor of viral RNA polymerase. *Proc Jpn Acad Ser B Phys Biol Sci*. (2017) 93:449–63. doi: 10.2183/pjab.93.027
39. Baranovich T, Wong SS, Armstrong J, Marjuki H, Webby RJ, Webster RG, et al. T-705 (favipiravir) induces lethal mutagenesis in influenza A H1N1 viruses *in vitro*. *J Virol*. (2013) 87:3741–751. doi: 10.1128/JVI.02346-12
40. Oestereich L, Lüdtke A, Wurr S, Rieger T, Muñoz-Fontela C, Günther S. Successful treatment of advanced Ebola virus infection with T-705 (favipiravir) in a small animal model. *Antiviral Res*. (2014) 105:17–21. doi: 10.1016/j.antiviral.2014.02.014
41. Yamada K, Noguchi K, Kimitsuki K, Kaimori R, Saito N, Komeno T, et al. Reevaluation of the efficacy of favipiravir against rabies virus using *in vivo* imaging analysis. *Antiviral Res*. (2019) 172:104641. doi: 10.1016/j.antiviral.2019.104641
42. Chen C, Huang J, Cheng Z, Wu J, Chen S, Zhang Y, et al. Favipiravir versus arbidol for COVID-19: a randomized clinical trial. *MedRxiv [Preprint]*. (2020) doi: 10.1101/2020.03.17.20037432

43. Du YX, Chen XP. Favipiravir: pharmacokinetics and concerns about clinical trials for 2019-nCoV infection. *Clin Pharmacol Ther*. (2020) 108:242–7.doi: 10.1002/cpt.1844
44. Maag D, Castro C, Hong Z, Cameron CE. Hepatitis C virus RNA-dependent RNA polymerase (NS5B) as a mediator of the antiviral activity of ribavirin. *J Biol Chem*. (2001) 276:46094–8. doi: 10.1074/jbc.C100349200
45. Crotty S, Cameron CE, Andino R. RNA virus error catastrophe: direct molecular test by using ribavirin. *Proc Natl Acad Sci USA*. (2001) 98:6895–900. doi: 10.1073/pnas.111085598
46. Zhou S, Liu R, Baroudy BM, Malcolm BA, Reyes GR. The effect of ribavirin and IMPDH inhibitors on hepatitis C virus subgenomic replicon RNA. *Virology*. (2003) 310:333–42. doi: 10.1016/S0042-6822(03)00152-1
47. Chemello L, Cavalletto L, Bernardinello E, Guido M, Pontisso P, Alberti A. The effect of interferon alfa and ribavirin combination therapy in naive patients with chronic hepatitis C. *J Hepatol*. (1995) 23(Suppl. 2):8–12.
48. Marcelin JR, Wilson JW, Razonable RR, Mayo Clinic Hematology/Oncology and Transplant Infectious Diseases Services. Oral ribavirin therapy for respiratory syncytial virus infections in moderately to severely immunocompromised patients. *Transpl Infect Dis*. (2014) 16:242–50. doi: 10.1111/tid.12194
49. Lee N, Hui D, Wu A, et al. A major outbreak of severe acute respiratory syndrome in Hong Kong. *N Engl J Med*. (2003) 348:1986–94. doi: 10.1056/NEJMoa030685
50. Booth CM, Matukas LM, Tomlinson GA, Rachlis AR, Rose DB, Dwosh HA, et al. Clinical features and short-term outcomes of 144 patients with SARS in the greater Toronto area. *JAMA*. (2003) 289:2801–9. doi: 10.1001/jama.289.21.JOC30885
51. So LK, Lau AC, Yam LY, Cheung TMT, Poon E, Yung RWH, et al. Development of a standard treatment protocol for severe acute respiratory syndrome. *Lancet*. (2003). 361:1615–7. doi: 10.1016/S0140-6736(03)13265-5
52. Morgenstern B, Michaelis M, Baer PC, Doerr HW, Cinatl J. Ribavirin and interferon-beta synergistically inhibit SARS-associated coronavirus replication in animal and human cell lines. *Biochem Biophys Res Commun*. (2005) 326:905–8. doi: 10.1016/j.bbrc.2004.11.128
53. Wang WK, Chen SY, Liu IJ, Kao CL, Chen HL, Chiang BL, et al. Temporal relationship of viral load, ribavirin, interleukin (IL)-6, IL-8, and clinical progression in patients with severe acute respiratory syndrome. *Clin Infect Dis*. (2004) 39:1071–5. doi: 10.1086/423808
54. Chen F, Chan KH, Jiang Y, Kao RY, Lu HT, Fan KW, et al. *In vitro* susceptibility of 10 clinical isolates of SARS coronavirus to selected antiviral compounds. *J Clin Virol*. (2004) 31:69–75. doi: 10.1016/j.jcv.2004.03.003
55. Omrani AS, Saad MM, Baig K, Bahloul A, Abdul-Matin M, Alaidaroos AY, et al. Ribavirin and interferon alfa-2a for severe middle East respiratory syndrome coronavirus infection: a retrospective cohort study. *Lancet Infect Dis*. (2014) 14:1090–5. doi: 10.1016/S1473-3099(14)70920-X
56. Zhao Z, Zhang F, Xu M, Huang K, Zhong W, Cai W, et al. Description and clinical treatment of an early outbreak of severe acute respiratory syndrome (SARS) in Guangzhou, PR China. *J Med Microbiol*. (2003) 52(Pt 8):715–20. doi: 10.1099/jmm.0.05320-0
57. Leong HN, Ang B, Earnest A, Teoh C, Xu W, Leo YS. Investigational use of ribavirin in the treatment of severe acute respiratory syndrome, Singapore, 2003. *Trop Med Int Health*. (2004) 9:923–7. doi: 10.1111/j.1365-3156.2004.01281.x
58. Khalid M, Al Rabiah F, Khan B, Al Mobeireek A, Butt TS, Al Mutairy E. Ribavirin and interferon-α2b as primary and preventive treatment for Middle East respiratory syndrome coronavirus: a preliminary report of two cases. *Antivir Ther*. (2015) 20:87–91. doi: 10.3851/IMP2792
59. Stockman LJ, Bellamy R, Garner P. SARS: systematic review of treatment effects. *PLoS Med*. (2006) 3:e343. doi: 10.1371/journal.pmed.0030343
60. Morra ME, van Thanh L, Kamel MG, Ghazy AA, Altibi AMA, Dat LM, et al. Clinical outcomes of current medical approaches for middle East respiratory syndrome: a systematic review and meta-analysis. *Rev Med Virol*. (2018) 28:e1977. doi: 10.1002/rmv.1977
61. Elfiky AA. Anti-HCV, nucleotide inhibitors, repurposing against COVID-19. *Life Sci*. (2020) 248:117477. doi: 10.1016/j.lfs.2020.117477
62. Dong L, Hu S, Gao J. Discovering drugs to treat coronavirus disease 2019. (COVID-19). *Drug Discov Ther*. (2020). 14:58–60. doi: 10.5582/ddt.2020.01012
63. Elfiky AA. Ribavirin, remdesivir, sofosbuvir, galidesivir, and tenofovir against SARS-CoV-2 RNA dependent RNA polymerase (RdRp): a molecular docking study. *Life Sci*. (2020) 253:117592. doi: 10.1016/j.lfs.2020.117592
64. Caly L, Druce JD, Catton MG, Jans DA, Wagstaff KM. The FDA-approved drug ivermectin inhibits the replication of SARS-CoV-2 *in vitro*. *Antiviral Res*. (2020) 178:104787. doi: 10.1016/j.antiviral.2020.104787
65. Mastrangelo E, Pezzullo M, de Burghgraeve T, Kaptein S, Pastorino B, Dallmeier K, et al. Ivermectin is a potent inhibitor of flavivirus replication specifically targeting NS3 helicase activity: new prospects for an old drug. *J Antimicrob Chemother*. (2012) 67:1884–94. doi: 10.1093/jac/dks147
66. Wagstaff KM, Sivakumaran H, Heaton SM, Harrich D, Jans DA. Ivermectin is a specific inhibitor of importin α/β-mediated nuclear import able to inhibit replication of HIV-1 and dengue virus. *Biochem J*. (2012) 443:851–6. doi: 10.1042/BJ20120150
67. Tay MY, Fraser JE, Chan WK, Moreland NJ, Rathore AP, Wang C, et al. Nuclear localization of dengue virus (DENV) 1-4 non-structural protein 5; protection against all 4 DENV serotypes by the inhibitor Ivermectin. *Antiviral Res*. (2013). 99:301–6. doi: 10.1016/j.antiviral.2013.06.002
68. Galeotti C, Kaveri SV, Bayry J. IVIG-mediated effector functions in autoimmune and inflammatory diseases. *Int Immunol*. (2017) 29:491–8. doi: 10.1093/intimm/dxx039
69. Jolles S, Sewell WA, Misbah SA. Clinical uses of intravenous immunoglobulin. *Clin Exp Immunol*. (2005) 142:1–11. doi: 10.1111/j.1365-2249.2005.02834.x
70. Kaveri SV, Maddur MS, Hegde P, Lacroix-Desmazes S, Bayry J. Intravenous immunoglobulins in immunodeficiencies: more than mere replacement therapy. *Clin Exp Immunol*. (2011) 164(Suppl. 2):2–5. doi: 10.1111/j.1365-2249.2011.04387.x
71. Bayry J, Lacroix-Desmazes S, Kazatchkine MD, Kaveri SV. Intravenous immunoglobulin for infectious diseases: back to the pre-antibiotic and passive prophylaxis era? *Trends Pharmacol Sci*. (2004) 25:306–10. doi: 10.1016/j.tips.2004.04.002
72. Ben-Nathan D, Lustig S, Tam G, Robinzon S, Segal S, Rager-Zisman B. Prophylactic and therapeutic efficacy of human intravenous immunoglobulin in treating West Nile virus infection in mice. *J Infect Dis*. (2003) 188:5–12. doi: 10.1086/376870
73. Jiang S, Hillyer C, Du L. Neutralizing antibodies against SARS-CoV-2 and other human coronaviruses. *Trends Immunol*. (2020) 41:355–9. doi: 10.1016/j.it.2020.03.007
74. Jawhara S. Could intravenous immunoglobulin collected from recovered coronavirus patients protect against COVID-19 and strengthen the immune system of new patients? *Int J Mol Sci*. (2020) 21:2272. doi: 10.3390/ijms21072272
75. Shen C, Wang Z, Zhao F, Yang Y, Li J, Yuan J, et al. Treatment of 5 critically Ill patients with COVID-19 with convalescent plasma. *JAMA*. (2020) 323:1582–9. doi: 10.1001/jama.2020.4783
76. Roback JD, Guarner J. Convalescent plasma to treat COVID-19: possibilities and challenges. *JAMA*. (2020) doi: 10.1001/jama.2020.4940. [Epub ahead of print].
77. Russell B, Moss C, George G, Santaolalla A, Cope A, Papa S, et al. Associations between immune-suppressive and stimulating drugs and novel COVID-19-a systematic review of current evidence. *Ecancermedicalscience*. (2020) 14:1022. doi: 10.3332/ecancer.2020.1022
78. Chihrin S, Loutfy MR. Overview of antiviral and anti-inflammatory treatment for severe acute respiratory syndrome. *Expert Rev Anti Infect Ther*. (2005) 3:251–62. doi: 10.1586/14787210.3.2.251
79. Lam CW, Chan MH, Wong CK. Severe acute respiratory syndrome: clinical and laboratory manifestations. *Clin Biochem Rev*. (2004) 25:121–32.
80. Zhang X, Alekseev K, Jung K, Vlasova A, Hadya N, Saif LJ. Cytokine responses in porcine respiratory coronavirus-infected pigs treated with corticosteroids as a model for severe acute respiratory syndrome. *J Virol*. (2008) 82:4420–8. doi: 10.1128/JVI.02190-07
81. Qin YY, Zhou YH, Lu YQ, Sun F, Yang S, Harypursat V, et al. Effectiveness of glucocorticoid therapy in patients with severe coronavirus disease 2019: protocol of a randomized controlled trial. *Chin Med J (Engl)*. (2020). 133:1080–6. doi: 10.1097/CM9.0000000000000791

82. Totura AL, Bavari S. Broad-spectrum coronavirus antiviral drug discovery. *Expert Opin Drug Discov*. (2019) 14:397–412. doi: 10.1080/17460441.2019.1581171
83. Ströher U, DiCaro A, Li Y, Strong JE, Aoki F, Plummer F, et al. Severe acute respiratory syndrome-related coronavirus is inhibited by interferon- alpha. *J Infect Dis*. (2004) 189:1164–7. doi: 10.1086/382597
84. Falzarano D, de Wit E, Martellaro C, Callison J, Munster VJ, Feldmann H. Inhibition of novel β coronavirus replication by a combination of interferon-α2b and ribavirin. *Sci Rep*. (2013) 3:1686. doi: 10.1038/srep01686
85. Falzarano D, de Wit E, Rasmussen AL, Feldmann F, Okumura A, Scott DP, et al. Treatment with interferon-α2b and ribavirin improves outcome in MERS-CoV-infected rhesus macaques. *Nat Med*. (2013) 19:1313–7. doi: 10.1038/nm.3362
86. Cinatl J, Morgenstern B, Bauer G, Chandra P, Rabenau H, Doerr HW. Treatment of SARS with human interferons. *Lancet*. (2003). 362:293–4. doi: 10.1016/S0140-6736(03)13973-6
87. Sallard E, Lescure FX, Yazdanpanah Y, Mentre F, Peiffer-Smadja N. Type 1 interferons as a potential treatment against COVID-19. *Antiviral Res*. (2020) 178:104791. doi: 10.1016/j.antiviral.2020.104791
88. Kopecky-Bromberg SA, Martínez-Sobrido L, Frieman M, Baric RA, Palese P. Severe acute respiratory syndrome coronavirus open reading frame (ORF) 3b, ORF 6, and nucleocapsid proteins function as interferon antagonists. *J Virol*. (2007) 81:548–57. doi: 10.1128/JVI.01782-06
89. Lokugamage KG, Hage A, Schindewolf C, Rajsbaum R, Menachery VD. SARS-CoV-2 is sensitive to type I interferon pretreatment. *bioRxiv [Preprint]*. (2020). doi: 10.1101/2020.03.07.982264
90. Shen KL, Yang YH. Diagnosis and treatment of 2019 novel coronavirus infection in children: a pressing issue. *World J Pediatr*. (2020) 16:219–21. doi: 10.1007/s12519-020-00344-6. [Epub ahead of print].
91. Zeng YM, Xu XL, He XQ, Tang SQ, Li Y, Huang YQ, et al. Comparative effectiveness and safety of ribavirin plus interferon-alpha, lopinavir/ritonavir plus interferon-alpha, and ribavirin plus lopinavir/ritonavir plus interferon-alpha in patients with mild to moderate novel coronavirus disease 2019: study protocol. *Chin Med J (Engl)*. (2020) 133:1132–4. doi: 10.1097/CM9.0000000000000790
92. Liu B, Li M, Zhou Z, Guan X, Xiang Y. Can we use interleukin-6 (IL-6) blockade for coronavirus disease 2019 (COVID-19)-induced cytokine release syndrome (CRS)? *J Autoimmun*. (2020) 111:102452. doi: 10.1016/j.jaut.2020.102452
93. Channappanavar R, Perlman S. Pathogenic human coronavirus infections: causes and consequences of cytokine storm and immunopathology. *Semin Immunopathol*. (2017) 39:529–39. doi: 10.1007/s00281-017-0629-x
94. Zhou Y, Fu B, Zheng X, Wang D, Zhao C, Qi Y, et al. Pathogenic T-cells and inflammatory monocytes incite inflammatory storms in severe COVID-19 patients. *Natl Sci Rev*. (2020) 7:998–1002. doi: 10.1093/nsr/nwaa041
95. Chen L, Liu HG, Liu W, Liu J, Liu K, Shang J, et al. [Analysis of clinical features of 29 patients with 2019 novel coronavirus pneumonia]. *Zhonghua Jie He He Hu Xi Za Zhi*. (2020). 43:E005. doi: 10.3760/cma.j.issn.1001-0939.2020.0005
96. Ruan Q, Yang K, Wang W, Jiang L, Song J. Clinical predictors of mortality due to COVID-19 based on an analysis of data of 150 patients from Wuhan, China. *Intensive Care Med*. (2020) 46:846–8. doi: 10.1007/s00134-020-05991-x
97. Zhang C, Wu Z, Li JW, Zhao H, Wang GQ. Cytokine release syndrome in severe COVID-19: interleukin-6 receptor antagonist tocilizumab may be the key to reduce mortality. *Int J Antimicrob Agents*. (2020) 55:105954. doi: 10.1016/j.ijantimicag.2020.105954
98. Xu X, Han M, Li T, Sun W, Wang D, Fu B, et al. Effective treatment of severe COVID-19 patients with tocilizumab. *Proc Natl Acad Sci USA*. (2020) 117:10970–5. doi: 10.1073/pnas.2005615117
99. Luo P, Liu Y, Qiu L, Liu X, Liu D, Li J. Tocilizumab treatment in COVID-19: a single center experience. *J Med Virol*. (2020) 92:814–8. doi: 10.1002/jmv.25801
100. Zhang X, Song K, Tong F, Fei M, Guo H, Lu Z, et al. First case of COVID-19 in a patient with multiple myeloma successfully treated with tocilizumab. *Blood Adv*. (2020) 4:1307–10. doi: 10.1182/bloodadvances.2020001907
101. Michot JM, Albiges L, Chaput N, Saada V, Pommeret F, Griscelli F, et al. Tocilizumab, an anti-IL-6 receptor antibody, to treat COVID-19-related respiratory failure: a case report. *Ann Oncol*. (2020) 31:961–4. doi: 10.1016/j.annonc.2020.03.300
102. de Luna G, Habibi A, Deux JF, Colard M, Pham Hung d&#39, Alexandry d&#39, et al. Rapid and severe Covid-19 pneumonia with severe acute chest syndrome in a sickle cell patient successfully treated with tocilizumab. *Am J Hematol*. (2020) 95:876–8. doi: 10.1002/ajh.25833
103. ClinicalTrials.gov. *National Library of Medicine (U.S)*. (2020). *Identifier: NCT04341389 A phase II Trial to evaluate the Recombinant Vaccine for COVID-19 (Adenovirus Vector) (CTII-nCoV)*. Available online at: https://clinicaltrials.gov/ct2/show/NCT04341389 (accessed June 24, 2020).
104. ClinicalTrials.gov. *National Library of Medicine (U.S)*. (2020). *Identifier: NCT04400838 Investigating a Vaccine against COVID-19*. Available online at: https://clinicaltrials.gov/ct2/show/NCT04400838?term=vaccine&cond=covid-19&phase=2&draw=2&rank=8 (accessed June 24, 2020).

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Communication

PERSPECTIVE
published: 31 July 2020
doi: 10.3389/fcomm.2020.00063

Addressing COVID-19 Communication and Management by a Systems Thinking Approach

Francesco Gonella[1,2]*, *Marco Casazza*[3], *Silvio Cristiano*[4] and *Alessandra Romano*[3,5]

[1] Department of Molecular Sciences and Nanosystems, Ca' Foscari Venice University, Venice, Italy, [2] Research Institute for Complexity, Ca' Foscari Venice University, Venice, Italy, [3] Division of Hematology, AOU "Policlinico—Vittorio Emanuele", Catania, Italy, [4] Department of Science and Technology, Università degli Studi di Napoli Parthenope, Naples, Italy, [5] Scuola Superiore di Catania, Università degli Studi di Catania, Catania, Italy

A systemic stock-flow diagram is proposed for the communication and management of health services and strategies concerning the COVID-19 epidemic. The possible role of government interventions in activating systemic leverage points is also addressed. The presented approach, based on Systems Thinking, can create the basis for creating an analytical simulator of the disease spread, and at the same time the diagram can constitute a powerful tool for improving the quality of information for both policy-makers and the general public in situations of epidemics.

Keywords: systems thinking, COVID-19, epidemics, communication, publich health

OPEN ACCESS

Edited by:
Mohan Jyoti Dutta,
Massey University, New Zealand

Reviewed by:
Margaret Usha D'Silva,
University of Louisville, United States
Qian Hu,
University of Central Florida, United States

***Correspondence:**
Francesco Gonella
gonella@unive.it

Specialty section:
This article was submitted to Health Communication, a section of the journal Frontiers in Communication

Received: *16 April 2020*
Accepted: *15 July 2020*
Published: *31 July 2020*

Citation:
Gonella F, Casazza M, Cristiano S and Romano A (2020) Addressing COVID-19 Communication and Management by a Systems Thinking Approach. Front. Commun. 5:63. doi: 10.3389/fcomm.2020.00063

INTRODUCTION

The organization and efficiency of health services has recently drawn considerable attention due to the COVID-19 pandemic (Gates, 2020). A lack of resources has been evidenced worldwide in the reaction toward the coronavirus spread (Jones, 2020). Diverting medical personnel and equipment from the cure of other pathologies created further malfunctioning in hospital services and in domestic assistance (Horton, 2020). A sense of precariousness and a lack of foresight have emerged, especially for prompt reaction measures taken by governance bodies (ILOSTAT, 2019), and are independent of the overall efficiency of healthcare systems in non-emergency situations. The Global Risk Report 2020 (World Economic Forum, 2020), published annually by the World Economic Forum, warned of this situation a few months ago, reporting (Nuclear Threat Initiative, 2019) that: "A recent first-of-its-kind comprehensive assessment of health security and related capabilities across 195 countries found fundamental weaknesses around the world: no country is fully prepared to handle an epidemic or pandemic." To identify the most effective interventions to halt the virus spread in its earliest stage is therefore a priority, but a systemic assessment of the emergency is still lacking. The main purpose of this paper is to address how the novel application of a methodological approach might be of immediate use for communication purposes of epidemics. The presented general diagram may be used by managers or decision-makers to address the problem of communication and emergency management at different levels, representing a powerful tool in the process of daily reporting of the situation to both the general public and operative stakeholders, as well as potentially contributing to the general improvement of scientific literacy.

SYSTEMIC APPROACHES TO HEALTHCARE

In the last two decades, new approaches have started catching on in the field of healthcare management. They aim at describing healthcare systems from an integrated, holistic point of view [for a general review on this field, see Carey et al. (2015)], calling for a stronger integration of systemic thinking into public health procedures and management (Fahey et al., 2004; Williams et al., 2005; Midgley, 2006; Trochim et al., 2006; Leischow et al., 2008; Mabry et al., 2008; Barabási et al., 2011; Hood et al., 2012; Wolkenhauer et al., 2013; Bishai et al., 2014; Peters, 2014). The World Health Organization itself produced a report entitled "Systems Thinking for health systems strengthening" (de Savigny and Taghreed, 2009). The various methods are usually based on computational tools, derived from social network analysis (SNA) procedures and the concurrent availability of very large sets of data, whereas systems thinking quantitative approaches based on stock-flow diagrams remain unexplored so far [see discussions in Dammann et al. (2014) and Cassidy et al. (2019)]. Systemic approaches were also used in the study of specific epidemics, or, more generally, in the development of epidemiology discipline (Ritchie-Dunham and Méndez Galván, 1999; Xia et al., 2017). Verelst et al. (2016) and Walters et al. (2018) reported recent reviews on this field. Computational models of epidemics often follow data-driven metapopulation procedures (Balcan et al., 2010; Mari et al., 2017), yet without encompassing the spatial and temporal dynamics of infections at the global scale, due to the difficulty in having reliable and significant extended data (Walters et al., 2018). Only a few works report the use of a systemic perspective in the assessment of strategies to limit contagion spreading (Gumel et al., 2004; Ferguson et al., 2005; Araz, 2013), indicating the urgent need for putting together medical and epidemiological issues with management tools. This is a crucial point, since scientific information during the emergency must be reliable but at the same time feasible for both politicians and the general public (Rybniker and Fätkenheuer, 2020). Based on this background, models of the coronavirus spreading dynamics were reported in recent weeks, mostly based on the determination of parameters from existing reliable big data (Chinazzi et al., 2020; Gatto et al., 2020; Hellewell et al., 2020; Kucharski et al., 2020; Li et al., 2020; Read et al., 2020; Wang et al., 2020; Wu et al., 2020). A key point differentiates the stock-flow quantitative systemic approach from those addressed in the cited works: our diagram is not a "photograph" of a collection of the existing elements, but rather a representation of those elements that determines the system dynamics, mutually interacting by means of properly defined processes. It is important to clarify that in the conceptual framework of stock-flow ST the words "system" and "systemic" do not have the same meaning they assume in the Network Analysis contexts, which the cited approaches are mostly based on. While both NA and ST aim at describing a system as a whole, the former still relies on the knowledge of a proper collection of empirical parameters and on the availability of sophisticated mathematical and statistical tools. On the contrary, the ST approach starts from the identification of the minimum set of state extensive variables (stocks) necessary to model the flows and the feedback network that describes the configurations of the system dynamics. The worldwide reference model by Johns Hopkins University (https://systems.jhu.edu/research/public-health/ncov-model-2/) is a good example of this difference. It presents a stochastic metapopulation epidemic simulation, based on a global network of city-level populations connected by edges representing passenger air travel between cities. From an epistemological point of view, it represents exactly the kind of computational-based approach that is complementary to the ST-based one. It starts from local connections (edges) between many physically existing elements, while in our approach we have a limited number of elements (the stocks) necessary to represent the system state, connected by physically existing flows of the same elements of the stocks. Owing to the processes, they form a network of mutual relationships that ultimately determine the evolution dynamics. Nevertheless, ST and NA approaches are two faces of the same complexity, whose complementarity has enormous potential, as pointed out by Bielekova et al. (2014): "The integration of systems thinking with dynamic computational modeling can lead to the development of a 'virtual sandbox' in which researchers can utilize their creativity and intuition to try out and explore multiple different hypotheses and lines of investigation." In this paper, we propose a comprehensive descriptive framework, based on stock-flow symbolic language used in energy systems diagramming, suitable to be adapted and used at different scales and for different epidemics and site-specific situations. The presented general diagram, developed on the basis of the COVID-19 emergency, is suitable to be integrated in the current data-driven models. This may be used by managers or decision-makers to address the problem of communication at different levels and of emergency management, representing a powerful tool in the process of daily reporting of the situation to both the general public and operative stakeholders, also potentially contributing to the general improvement of scientific literacy.

SYSTEMS THINKING AND STOCK-FLOW DIAGRAMS

The Systems Thinking (ST) approach was developed from the pioneering work of Ludwig von Bertalanffy on General Systems Theory (von Bertalanffy, 1968). It has found applications in quite a wide range of fields, from hard sciences to sociology and economics. From an operational point of view, the first (and fundamental) step of an ST-based analysis is the creation of a systemic diagram containing all the relevant elements that define the system operation at the decided level of study (Bossel, 1994, 2007; Luna-Reyes and Andersen, 2003). The systemic diagram presented for COVID-19 was set up following four basic steps:

1. Identification of the set of variables suitable to describe the emergency evolution as a system.
   The variables must be *countable extensive state variables* that constitute an n-tuple of numbers that at any time represents *a state of the system*. In the system's language, these variables

are called the *stocks*. The number of stocks must be the minimum necessary to describe the state of the system for the prescribed purposes. It must be possible to describe the relevant processes occurring in the system in terms of stocks interactions. In the current case, we first chose stocks made of people at different stages of the disease, from healthy ones who become infected to either recovered or dead. These stocks are also needed to describe the epidemic from the point of view of the news reported every day by media, on which public perception is based. The second type of stocks represent those necessary to study the epidemic (local) management, so they include medical personnel, equipment, and devices, and the government, whose role is that of providing means and implementing suitable intervention measures to contain the disease spread.

2. Identification of the flows connecting the stocks and the external environment.
   A stock may change its value only upon its inflows and/or its outflows, represented by arrows entering or exiting the stock. If Q is the quantity of something contained in a stock, then inflows and outflows are expressed as dQ/dt.
3. Identification of the processes occurring within the system.
   Processes are mechanisms able to change the value of a flow. Since the system state is a collection of stocks, and the only way to change the value of a stock is by acting on its in/outflows, processes are located along the flows. Flows between stocks may have different natures, but are usually matter flows. A flow between a stock and a process may also represent a control, exerted by the stock on the process. It this case, the flow is made of either information or labor or services.
4. Identification of feedbacks.
   The value of a flow may alter the value of a stock, but if this change also alters the value of the flow in a cause-effect loop, we say we are in the presence of *feedback*. This may be direct or indirect, the latter of which is when the mutual change in the flow and stock values follows a path that includes other stocks. The pattern of feedback is the feature that defines the ultimate dynamics of a system (Meadows, 2008).

It is worth underlining that a stock-flow diagram is not a "photograph" of the system, but rather an abstract representation that has a mainly epistemological role, based on a description of the pattern of reciprocal influences between the stocks. The epistemology of modeling in systems thinking is very rigorous [see for example Odum and Odum (2000)], passing from a structural model (the diagram) to an analytical one (the set of differential equations), to a computational one (the simulator), the latter connecting the state of the system (the n-tuple of stock variables that defines a point in a state's space) to a point in the space-time diagram of the system evolution. This conceptual structuring is rarely addressed explicitly in the application of ST diagrams, but it constitutes the core of the use of this approach in several disciplines, such as ecology, hard sciences, economy, and anthropology, while its application in both communication and health management issues is, to our knowledge, almost completely unaddressed.

SYSTEMS THINKING AND COVID-19

Figure 1 shows the ST diagram of the COVID-19 spread. Symbols are borrowed from the energy language (Odum and Odum, 2000), where shields indicate the stocks, arrowed solid lines the flows, dashed solid lines the controls on processes, and solid big arrows the processes. Sick (K) elements are defined as people who have been positively diagnosed, and who will be either hospitalized or quarantined, while Symptomatic (Y) have not been diagnosed yet. This division in two different stocks is purely systemic, and is concerned only with our knowledge. It is made to specify the "Diagnosis" process in the diagram. In fact, this process requires resources in terms of medical equipment (e.g., swabs) and thus is a possible critical point for epidemic management. "Diagnosis" has therefore a systemic meaning; it is the process that makes a person who belongs to the stock Infected (IN) become an element of the stock Sick (K), independent of the type of diagnosis. The processes labeled with M (measure) represent the systemic locations of possible leverage points. In fact, these processes are the elements on which government measures and interventions may act, for instance, by imposing restrictions to contacts or by providing more resources in terms of equipment and labor-force of medical personnel. They are based on the elaboration of the information flow exiting the system. As already remarked, the stock-flow diagram was prepared first of all by creating the stocks of people at different stages of the disease or differently treated. Other stocks were then included, necessary to point out the processes involved in the epidemic management. Among these, sanitary personal devices (SPD) played a role in weakening the reinforcing feedback in the Infected (IN) stock, while the medical equipment stock (ME) was necessary for both the "Diagnosis" and the "Cure" processes. The medical personnel are more likely to be infected by symptomatics (through the "Contagion" process), since doctors and nurses work in contact with them, whereas healthy people are more likely to be infected by people from the Infected (IN) stock, since they are generally expected to avoid contact with persons presenting symptoms. In the diagram, the controls exerted on some of the processes by governance management are indicated by dashed arrows, which are actually flows of information or services. The stocks and the flows chosen for this diagram can offer a synthetic and clear way to report to the public the relevant data that day by day are necessary to follow the epidemic evolution. **Figure 2** shows a simplified version of the diagram, keeping only the people stocks (except for the personnel) and the corresponding flows. This synopsis corresponds to the actual information typically given, day by day, by the government, various agencies, and the mass media, as reported in the corresponding legend within the figure. The figure also

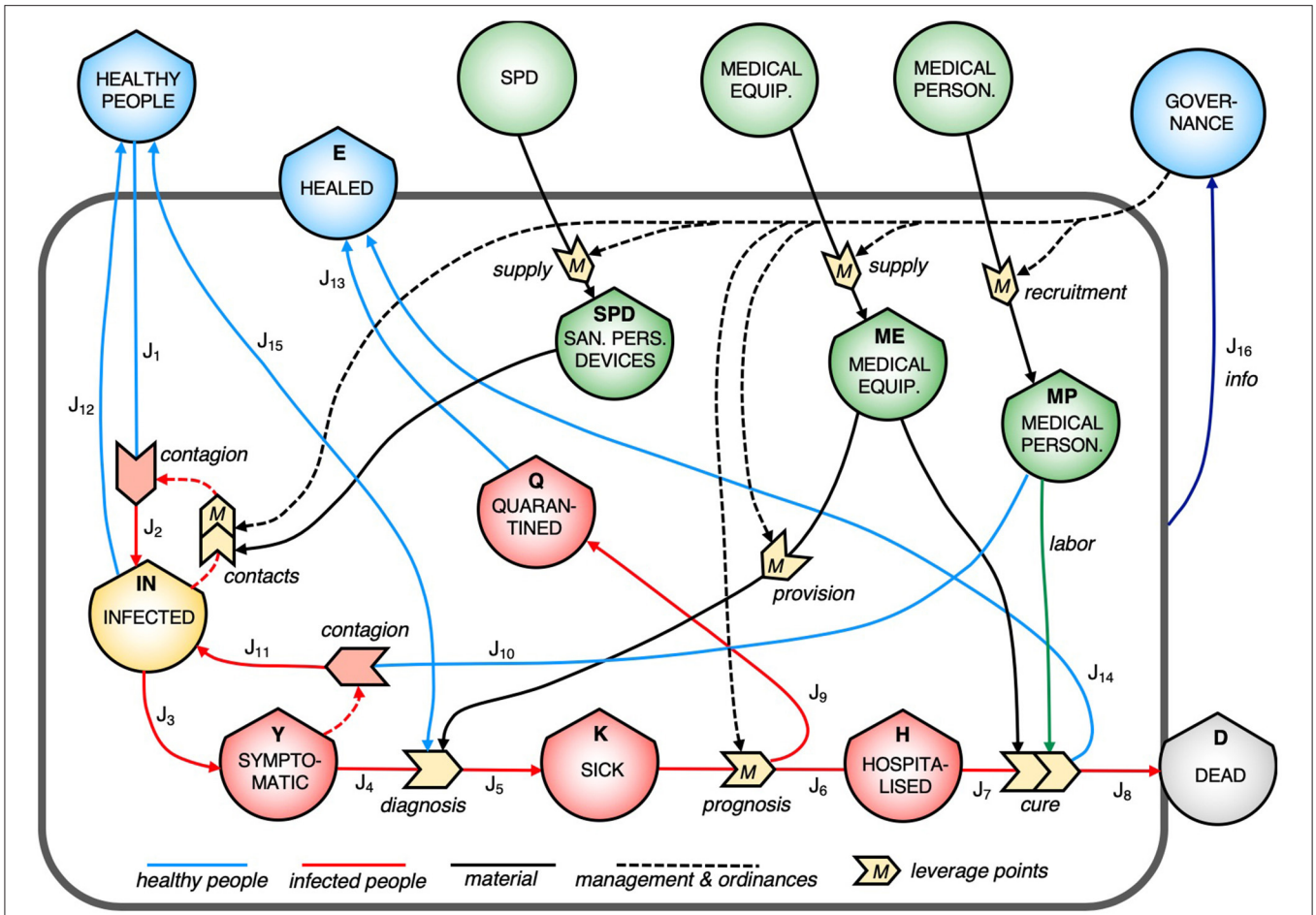
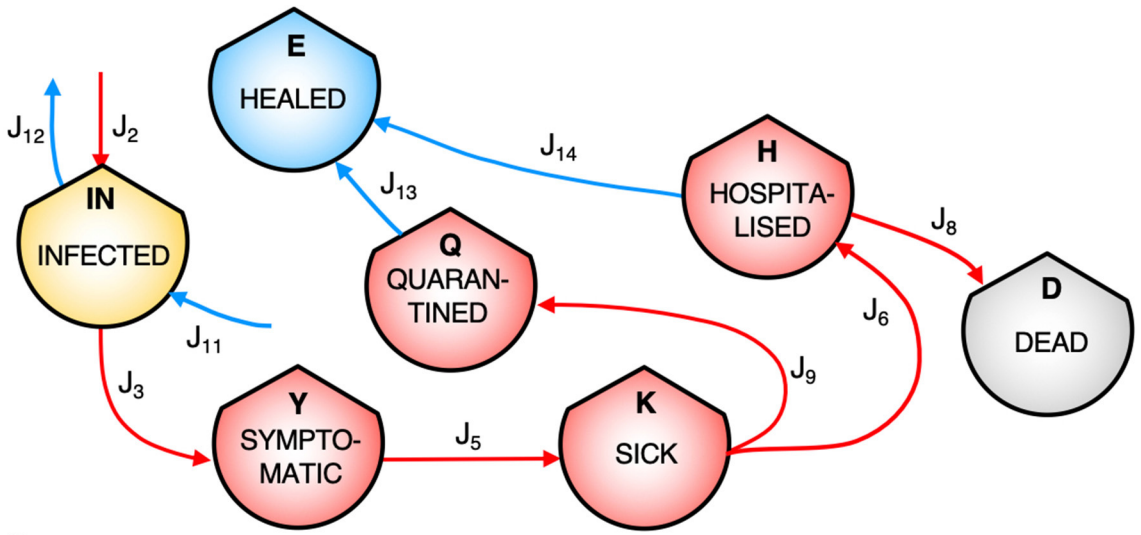


FIGURE 1 | Systems Thinking stock-flow diagram for the COVID-19 epidemic description. Stocks (shield symbols) are: IN, infected people whitout symptoms; Y, infected with symptoms; K, diagnosed sick people; Q, people in quarantine; H, hospitalized people; D, dead people; E, healed people (returned to normal); SPD, sanitary personal devices (face masks, gloves, disinfectant); ME, medical equipment (including swabs, test kits, personal protective equipment, and intensive care equipment, especially mechanical ventilators and oxygen). Blue and red arrowed lines (labeled with *J* for subsequent mathematical representation) are the flows of people in the system, that in turn define day by day the value of the people stocks and the systemic state of the system. Solid black lines are the flow of resources needed to take on the epidemic, with the green line representing the medical personnel labor. Dashed black lines indicate the governance measures implemented to face the crisis. From a systemic point of view, they are flows of information or services. Big arrowed symbols are the processes, labeled with "M" wherever they could represent an effective leverage point for intervention. Colors are: blue for healthy people and governance, red or yellow for infected people, and green for generic systemic resources.

reports the mathematical expressions of the corresponding system elements.

DISCUSSION AND CONCLUSION

This version of the diagram allows for the following of the flows of people exiting the diagnosis process to populate the stocks of Sick (K) and Healed (E) by the flows J_5 and J_{15}, respectively. The Infected (IN) stock is characterized by a reinforcing feedback, that through the process named "Contacts" may activate the exponential increase of infected people. The value of the stock Infected (IN) is virtually unknown, thus the strength of the feedback remains unknown as well, at least during the emergency state. Moreover, some infected people remain asymptomatic, eventually going back to the stock E without being counted (flow J_{12} in **Figure 1**). People from the stock K populate both the Quarantine (Q) and the Hospitalized (H) stocks, the latter undergoing the cure processes, in turn activated by the availability of medical equipment and by the labor of medical personnel, represented by the two flows entering the process. A special role is played by the infection of physicians and nurses coming from their interactions with infected people (transformation of the flow J_{10} into the flow J_{11}), since the depletion in their qualified labor provision is potentially crucial in determining the worsening of assistance, that will be systemically evidenced by an increase of the flow J_8.



Media information	Type of information	Expression
New positive (persons/day)	flow	$J_5 = J_2 + J_{11} - J_{12}$
New hospitalized (persons/day)	flow	$J_6 = J_5 - J_9$
Presently hospitalized (persons)	stock (H)	$H = \int (J_6 - J_8 - J_{14})\, dt$
New dead (persons/day)	flow	$dD/dt = J_8$
Total dead (persons)	stock (D)	$D = \int (J_8)\, dt$
New healed (persons/day)	flow	$dE/dt = J_{13} + J_{14}$
Total healed (persons)	stock (E)	$E = \int (J_{13} + J_{14})\, dt$
Presently sick (persons)	stock (K)	$K = \int (J_5 - J_6 - J_9)\, dt$
Overall positives diagnosed from epidemic start (persons)	stock	$E+D+H+Q = \int (J_5)\, dt$

FIGURE 2 | Simplified version of the diagram, keeping only people stocks and the corresponding flows. The figure visualizes the actual information typically given day by day by the media in terms of stocks and flows, as reported in the legend within the figure (J_9 may be assumed to be equal to J_{13}). The mathematical espressions of the elements are also given.

Six possible leverage points are present, represented by the dashed lines and the corresponding processes labeled with *M* (measure). Some of them (i.e., those entering in the "Recruitment," "Provision," and "Supply" processes) depend on the overall balancing feedback provided by the flow of information J_{16}, collected daily from the system state. The control on the process "Prognosis" is particularly delicate since, on one hand, hospitalization should guarantee a higher level of medical care, while, on the other hand, overcrowded structures make the risk of contagion increase. This is one of the crucial points to be determined; in fact, the domestic assistance quality should be increased in order to limit the proximity to infected people, but without compromising the overall quality of the assistance. The control on the "Contacts" process is also crucial, as demonstrated by the different evolutions of the pandemic in countries that have followed different lockdown strategies. Specific attention has to be paid to the related reinforcing feedback, since any of the possible levels of intervention in the process can be highly site-dependent (population density, cultural aspects, socio-economic factors, etc.) and should take into account the overall effect on the social structure. Generally speaking, this diagram further addresses the necessity of treating a complex situation using complex integrated tools, like those provided by the ST framwork. At this level, the presented systemic description is a general structure, and does not provide specific details on the biomedical mechanisms of contagion, nor on the epidemiological aspects. As a matter of fact, different social communities and infrastructures may exhibit relevant

differences in their systemic arrangement, and the disease spread depends on social, geographical, climatic, and political aspects, as well as on the local availability of appropriate resources. The ST diagram may be adapted and used at different scales and situations, with parameters and elements adjusted depending on the type of infection, the strength of the contagion, and the involved community. For example, the choice of the stocks to include in the diagram might require us to specify a stock of people who cannot undergo lockdown conditions, for example dwellers of overpopulated urban slums. In this case, the level of governance intervention should take into account the existence of sub-stocks of infected people that operate differently in the system, in order to tailor the measures for a better overall efficacy. A specific study including a finer description of these aspects goes beyond the scope of this paper, but the characteristics of our diagram still indicates the flexibility of our stock-flow approach in the description of different situations. By the definition of proper equations for the accumulation-discharge of each stock [see Odum and Odum (2000)], a simulator can be used to study the evolution of the system configurations, particularly under different emergency management interventions. Of course, once the quantitative simulator of the diagram is created, a validation process will be necessary. Both processes (simulator creation and validation) follow standard procedures described in Systems Thinking and System Dynamics textbooks, like, for example, the classical book by Bossel (1994). The development of an actual quantitative example of computer simulation is well-beyond the scope of the paper, that is not aimed at predicting any outcome, at least before the pandemic is exhausted and proper data are collected. This is due to the intrinsic complexity of the system, whose behavior quantification requires the knowledge of phenomenological parameters that can be estimated only when the epidemic will be virtually disappeared within the reference community at issue. In particular, some coefficients must be defined for measuring the effectiveness of controls by the government, represented by the dashed lines in our diagram. In fact, in all the described processes, they act as a multiplying factor able to either increase or decrease a physically real flow. These coefficients, together with medical parameters representing mortality, morbidity, incubation time, etc., cannot be presently estimated with a sufficiently low uncertainty, so that the exercise of running a simulator in the context of dynamical systems analysis (Sterman, 2000) would not yet provide a reliable prediction. The proposed general diagram, developed on the basis of the COVID-19 emergency, can be integrated on the current data-driven models, providing a tool to simulate the dynamics of different epidemics and to indicate the leverage points at the level and type of socio-sanitary interventions. It can be implemented and enriched depending on the level of complexity of the study at hand, as well as on the specific target of the study.

An important consideration must finally be made regarding the communicative potential of this ST-based diagram. In fact, the confusion between the concepts of stock and flow is a common example of the general lack of scientific literacy. In particular, the concept of "accumulation" (that is, the stock value) is often misunderstood even by well-educated people (Cronin et al., 2009). In the case of the epidemic, the daily evolution of a stock is often perceived as that of its inflow, thereby creating wrong perceptions about what is going on. For example, some stock values will never decrease [e.g., Healed (E) and Dead (D)] over time, while their derivatives (i.e., their inflows) may increase or decrease depending on the epidemic evolution. This also applies for the Sick (K) stock, whose value at a time may be much less important than the behavior of its inflow and outflow. The diagram we propose, along with the corresponding table (except for the mathematical expressions), may be a powerful communication tool, as far as it offers a standardized pictorial representation able to explain the tables of data and the graphics reported in the media communications. The graphical synopsis provided by the ST diagram may significantly help the dissemination of correct information about the epidemic's development to the general public, since it connects the reported figures and trends with the overall dynamics of the disease spread. The diagram helps to explain to an audience with a low level of scientific literacy the differences between cumulative numbers and daily trends, linked to stocks and flows, and how they are related to each other. More scientifically educated people should more easily be able to focus on the relationships between quantities and their derivatives, and appreciate what this means in terms of overall balances of the stocks. It is our opinion that all the communication sources should refer to a picture similar to that of **Figure 2**, helping to create a better understanding even of the epidemiological aspects involved in the epidemic. Once the structure of the system and the relevant processes are identified, their control will be crucial to guarantee a good systemic functioning, that may be in turn determined by proper data collection and scenario simulations. The diagram will be then used both as an analytical and a communication tool, able to evidence the causes of malfunctioning and the possible effective protocols to be activated for the system management.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

FG and AR had the idea, developed, and wrote the overall perspective contribution. MC and SC contributed to the bibliographic search and to the diagram set-up. All authors contributed to the article and approved the submitted version.

REFERENCES

Araz, O. M. (2013). Integrating complex system dynamics of pandemic influenza with a multi-criteria decision making model for evaluating public health strategies. *J. Systems Sci. Systems Eng.* 22, 319–339. doi: 10.1007/s11518-013-5220-y

Balcan, D., Gonçalves, B., Hu, H., Ramasco, J. J., Colizza, V., and Vespignani, A. (2010). Modeling the spatial spread of infectious diseases: the global epidemic and mobility computational model. *J. Comput. Sci.* 1, 132–145. doi: 10.1016/j.jocs.2010.07.002

Barabási, A.-L., Gulbahce, N., and Loscalzo, J. (2011). Network medicine: a network-based approach to human disease. *Nat. Rev. Genet*. 12, 56–68. doi: 10.1038/nrg2918

Bielekova, B., Vodovotz, Y., An, G., and Hallenbeck, J. (2014). How implementation of systems biology into clinical trials accelerates understanding of diseases. *Front. Neurol.* 5:102. doi: 10.3389/fneur.2014.00102

Bishai, D., Paina, L., Li, Q., Peters, D. H., and Hyder, A. A. (2014). Advancing the application of systems thinking in health: why cure crowds out prevention. *Health Res. Policy Syst*. 12:28. doi: 10.1186/1478-4505-12-28

Bossel, H. (1994). *Modeling and Simulations*. Wellesley, MA: AK Peters.

Bossel, H. (2007). *Systems and Models: Complexity, Dynamics, Evolution, Sustainability*. Norderstedt: Books on Demand.

Carey, G., Malbon, E., Carey, N., Joyce, A., Crammond, B., and Carey, A. (2015). Systems science and systems thinking for public health: a systematic review of the field. *BMJ Open* 5:e009002. doi: 10.1136/bmjopen-2015-009002

Cassidy, R., Singh, N. S., Schiratti, P.-R., Semwanga, A., Binyaruka, P., Sachingongu, N., et al. (2019). Mathematical modelling for health systems research: a systematic review of system dynamics and agent-based models. *BMC Health Services Res.* 19:845. doi: 10.1186/s12913-019-4627-7

Chinazzi, M., Davis, J. T., Ajelli, M., Gioannini, C., Litvinova, M., Merler, S., et al. (2020). The effect of travel restrictions on the spread of the 2019 novel coronavirus (COVID-19) outbreak. *Science* 6:eaba9757. doi: 10.1126/science.aba9757

Cronin, M. A., Gonzalez, C., and Sterman, J. D. (2009). Why don't well-educated adults understand accumulation? A challenge to researchers, educators, and citizens. *Org. Behav. Human Decision Processes* 108, 116–130. doi: 10.1016/j.obhdp.2008.03.003

Dammann, O., Gray, P., Gressens, P., Wolkenhauer, O., and Leviton, A. (2014). Systems epidemiology: what's in a name? *Online J. Public Health Inform.* 6:e198. doi: 10.5210/ojphi.v6i3.5571

de Savigny, D., and Taghreed, A. (2009). *Systems Thinking for Health Systems Strengthening*. Geneva: Alliance for Health Policy and Systems Research, World Health Organisation.

Fahey, D. K., Carson, E. R., Cramp, D. G., and Muir Gray, J. A. (2004). Applying systems modelling to public health. *Systems Res. Behav. Sci.* 21, 635–649. doi: 10.1002/sres.602

Ferguson, N. M., Cummings, D. A., Cauchemez, S., Fraser, C., Riley, S., Meeyai, A., et al. (2005). Strategies for containing an emerging influenza pandemic in Southeast Asia. *Nature* 437, 209–214. doi: 10.1038/nature04017

Gates, B. (2020). Responding to covid-19—a once-in-a-century pandemic? *N. Engl. J. Med*. 382, 1677–1679. doi: 10.1056/NEJMp2003762

Gatto, M., Bertuzzo, E., Mari, L., Miccoli, S., Carraro, L., Casagrandi, R., et al. (2020). *Spread Dynamics of the Covid-19 Epidemic in Italy: Effects of Emergency Containment Measures. PNAS [pre-print]*. Available online at: http://covid-19-italy.s3-website-eu-west-1.amazonaws.com/SPREAD_DYNAMICS_OF_THE_COVID-19_EPIDEMIC_IN_ITALY_preprint.pdf (accessed April 12, 2020).

Gumel, A. B., Ruan, S., Day, T., Watmough, J., Brauer, F., van den Driessche, P., et al. (2004). Modelling strategies for controlling SARS outbreaks. *Proc. Royal Soc. B* 271, 2223–2232. doi: 10.1098/rspb.2004.2800

Hellewell, J., Abbott, S., Gimma, A., Bosse, N. I., Jarvis, C. I., Russell, T. W., et al. (2020). Feasibility of controlling COVID-19 outbreaks by isolation of cases and contacts. *Lancet Global Health* 8, e488–e496. doi: 10.1016/ S2214-109X(20)30074-7

Hood, L., Balling, L., and Auffray, C. (2012). Revolutionizing medicine in the 21st century through systems approaches. *Biotechnol. J.* 7, 992–1001. doi: 10.1002/biot.201100306

Horton, R. (2020). Offline: don't let COVID-19 divert us completely. *Lancet* 395:1534. doi: 10.1016/S.0140-6736(20)31130-2

ILOSTAT, International Labour Organization (2019). *COVID-19: Are There Enough Health Workers?* Available online at: https://ilostat.ilo.org/covid-19-are-there-enough-health-workers/ (accessed May 18, 2020).

Jones, D. S. (2020). History in a crisis—lessons for covid-19. *N. Engl. J. Med*. 382, 1681–1683. doi: 10.1056/NEJMp2004361

Kucharski, A. J., Russell, T. W., Diamond, C., Liu, Y., Edmunds, J., Funk, S., et al. (2020). Early dynamics of transmission and control of COVID-19: a mathematical modelling study. *Lancet Inf. Dis.* 20, 553–558. doi: 10.1016/S1473-3099(20)30144-4

Leischow, S. J., Best, A., Trochim, W. M., Clark, P. I., Gallagher, R. S., Marcus, S. E., et al. (2008). Systems thinking to improve the public's health. *Am. J. Prev. Med.* 35, S196–S203. doi: 10.1016/j.amepre.2008.05.014

Li, Q., Guan, X., Wu, P., Wang, X., Zhou, L., Tong, Y., et al. (2020). Early transmission dynamics in Wuhan, China, of Novel Coronavirus-infected pneumonia. *N. Engl. J. Med*. 382, 1199–1207. doi: 10.1056/NEJMoa2001316

Luna-Reyes, L. F., and Andersen, D. L. (2003). Collecting and analyzing qualitative data for system dynamics: methods and models. *Syst. Dyn. Rev*. 19, 271–296. doi: 10.1002/sdr.280

Mabry, P. L., Olster, D. H., Morgan, G. D., and Abrams, D. B. (2008). Interdisciplinarity and systems science to improve population health. *Am. J. Prev. Med*. 35, S211–S224. doi: 10.1016/j.amepre.2008.05.018

Mari, L., Gatto, M., Ciddio, M., Dia, E. D., Sokolow, S. H., De Leo, G., et al. (2017). Big-data-driven modeling unveils country-wide drivers of endemic schistosomiasis. *Sci. Rep.* 7:489. doi: 10.1038/s41598-017-00493-1

Meadows, D. H. (2008). *Thinking in Systems: A Primer*. White River Junction, VT: Chelsea Green Publishing.

Midgley, G. (2006). Systemic intervention for public health. *Am. J. Public Health* 96, 466–472. doi: 10.2105/AJPH.2005.067660

Nuclear Threat Initiative (2019). *Global Health Security Index: Inaugural Global Health Security Index Finds No Country Is Prepared for Epidemics or Pandemics*. NTI Press Release. Available online at: https://www.nti.org/newsroom/news/inaugural-global-health-security-index-finds-no-country-prepared-epidemics-or-pandemics/ (accessed March 15, 2020).

Odum, H. T., and Odum, E. C. (2000). *Modeling for All Scales: An Introduction to System Simulation*. San Diego: Academic Press.

Peters, D. H. (2014). The application of systems thinking in health: why use systems thinking? *Health Res. Policy Sys*. 12:51. doi: 10.1186/1478-4505-12-51

Read, J. M., Bridgen, J. R., Cummings, D. A., Ho, A., and Jewell, C. P., (2020). Novel coronavirus 2019-nCoV: early estimation of epidemiological parameters and epidemic predictions. *medRxiv*. doi: 10.1101/2020:01:23:20018549

Ritchie-Dunham, J. L., and Méndez Galván, J. F. (1999). Evaluating epidemic intervention policies with systems thinking: a case study of dengue fever in Mexico. *System Dyn. Rev.* 15, 119–138. doi: 10.1002/(SICI)1099-1727(199922)15:2<119::AID-SDR163>3.0.CO;2-G

Rybniker, J., and Fätkenheuer, G. (2020). Importance of precise data on SARS-CoV-2 transmission dynamics control. *Lancet*. doi: 10.1016/S1473-3099(20)30359-5. [Epub ahead of print].

Sterman, J. D. (2000). *Business Dynamics: Systems Thinking and Modeling for a Complex World*. New York, NY: McGraw-Hill.

Trochim, W. M., Cabrera, D. A., Milstein, B., Gallagher, R. S., and Leischow, S. J. (2006). Practical challenges of systems thinking and modeling in public health. *Am. J. Public Health* 96:538. doi: 10.2105/AJPH.2005.066001

Verelst, F., Willem, L., and Beutels, P. (2016). Behavioural change models for infectious disease transmission: a systematic review (2010–2015). *J. R. Soc. Interface* 13:20160820. doi: 10.1098/rsif.2016.0820

von Bertalanffy, L. (1968). *General System Theory: Foundations, Development, Applications*. New York, NY: George Braziller.

Walters, C. E., Meslé, M. M. I., and Halla, I. M. (2018). Modelling the global spread of diseases: a review of current practice and capability. *Epidemics* 25, 1–8. doi: 10.1016/j.epidem.2018.05.007

Wang, W., Tang, J., and Wei, F. (2020). Updated understanding of the outbreak of 2019 novel coronavirus (2019-nCoV) in Wuhan, China. *J. Med. Virol.* 92, 441–447. doi: 10.1002/jmv.25689

Williams, W., Lyalin, D., and Wingo, P. A. (2005). Systems thinking: what business modeling can do for public health. *J. Public Health Manag. Pract.* 11, 550–553. doi: 10.1097/00124784-200511000-00012

Wolkenhauer, O., Auffray, C., Jaster, R., Steinhoff, G., and Dammann, O. (2013). The road from systems biology to systems medicine. *Pediatric Res.* 73, 502–507. doi: 10.1038/pr.2013.4

World Economic Forum (2020). *Global Risk Report.* Available online at: https://www.weforum.org/reports/the-global-risks-report-2020 (accessed March 15, 2020).

Wu, J. T., Leung, K., and Leung, G. M. (2020). Nowcasting and forecasting the potential domestic and international spread of the 2019-nCoV outbreak originating in Wuhan, China: a modelling study. *Lancet* 395, 689–697. doi: 10.1016/S0140-6736(20)30260-9

Xia, S., Zhou, X.-N., and Liu, J. (2017). Systems thinking in combating infectious diseases. *Infect Dis. Poverty* 6:144. doi: 10.1186/s40249-017-0339-6

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pediatrics

BRIEF RESEARCH REPORT
published: 31 July 2020
doi: 10.3389/fped.2020.00491

Quarantine Due to the COVID-19 Pandemic From the Perspective of Pediatric Patients With Type 1 Diabetes: A Web-Based Survey

Stefano Passanisi[1]*, *Maria Pecoraro*[1], *Francesco Pira*[2], *Angela Alibrandi*[3], *Vittoria Donia*[1], *Paola Lonia*[1], *Giovanni Battista Pajno*[1], *Giuseppina Salzano*[1] and *Fortunato Lombardo*[1]

[1] Department of Human Pathology in Adult and Developmental Age "Gaetano Barresi", University of Messina, Messina, Italy, [2] Department of Ancient and Modern Civilizations, University of Messina, Messina, Italy, [3] Unit of Statistical and Mathematical Sciences, Department of Economics, University of Messina, Messina, Italy

Background: A crucial aspect of the 2019 coronavirus disease (COVID-19) pandemic was the psychological impact on the population. Most countries issued restrictive laws to reduce community-based viral spread. Children and adolescents were forced to experience physical and social distancing. Subjects with chronic diseases, such as type 1 diabetes, were more vulnerable and at higher risk of developing psychological disorders.

Methods: We conducted a web-based survey to investigate the behavioral responses during quarantine due to the COVID-19 outbreak in a cohort of pediatric patients with type 1 diabetes. Data were collected on demographic and clinical characteristics, lifestyle changes, and the impact of COVID-19 on the management of diabetes.

Results: Two hundred four pediatric patients (aged 5–18 years) with type 1 diabetes completed the questionnaire. Interestingly, patients ≤12 years were significantly more influenced by the quarantine period in their approach to the disease than older patients.

Conclusion: Although quarantine was a stressful psychological condition, our results showed that most children and adolescents with type 1 diabetes developed high levels of resilience and excellent coping skills by using technology in a proper way.

Keywords: children, coronavirus, lockdown, outbreak, management, resilience, technology

OPEN ACCESS

Edited by:
Lorenzo Iughetti, University of Modena and Reggio Emilia, Italy

Reviewed by:
Danilo Buonsenso, Catholic University of the Sacred Heart, Italy
Maurizio Delvecchio, Giovanni XXIII Children's Hospital, Italy

***Correspondence:**
Stefano Passanisi
spassanisi87@gmail.com

Specialty section:
This article was submitted to Pediatric Infectious Diseases, a section of the journal Frontiers in Pediatrics

Received: *14 May 2020*
Accepted: *14 July 2020*
Published: *31 July 2020*

Citation:
Passanisi S, Pecoraro M, Pira F, Alibrandi A, Donia V, Lonia P, Pajno GB, Salzano G and Lombardo F (2020) Quarantine Due to the COVID-19 Pandemic From the Perspective of Pediatric Patients With Type 1 Diabetes: A Web-Based Survey. Front. Pediatr. 8:491. doi: 10.3389/fped.2020.00491

INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, also known as 2019 Coronavirus disease (COVID-19), erupted in China on December 2019 and spread worldwide in very few months (1). Italy is currently one the most affected countries in Europe. The World Health Organization on March 11, 2020 declared COVID-19 a pandemic. The most direct and dramatic consequence of this pandemic is the high number of deaths due to SARS-CoV-2 infection. At the time of this publication 511,909 subjects died all over the world (2). A crucial, underestimated aspect is the heavy psychological impact on the population. Most countries issued strict governmental decrees that imposed self-isolation and social distancing in order to minimize community-based viral transmission. In Italy, the lock-down period lasted from March 9 to May 3, 2020 for a total of 55 days. Hospital shut their outpatient services, deferring all "non-urgent"

healthcare activities (3). These restrictive measures led to a gradual reduction of new cases of infection (4). However, people were forced to radically change their daily lifestyles and were at high risk of developing feelings of panic, anxiety, depression, and sometimes even dread (5). The psychological aspects of the COVID-19 pandemic also influenced the pediatric population due to the strong experience of physical and social isolation (6). In addition, children and adolescents suffering from type 1 diabetes (T1D) were unable to comply with scheduled outpatient follow-up visits and were also forced to modify the approach to the management of their chronic disease.

Aim of this study was to investigate the behavioral responses during the quarantine due to the COVID-19 pandemic in a cohort of Italian pediatric patients with T1D.

MATERIALS AND METHODS

From April 15 to May 1, 2020 we conducted a cross-sectional survey based on an on-line questionnaire. We enrolled 204 children and adolescents (aged 5–18 years) diagnosed with T1D for at least 3 months, and followed-up at our Pediatric Diabetes Centre in Messina. The online link for the questionnaire was sent to one of the parents and to the patient itself if over 12 years, and they were encouraged to fill it out together. Written informed consent through on-line form was obtained from patients' parents. The study was conducted in accordance with the Helsinki Declaration. The questionnaire included fourteen questions focusing on patients' demographic and clinical characteristics (e.g., age, gender, diabetes duration, insulin regimen, type of glucose monitoring), lifestyle changes during the quarantine period, and the impact of the lock-down on the management of diabetes. Finally, the participants were asked to quantify how much the quarantine influenced their approach to the disease according to the following four response levels: no influence, poor influence, relevant influence, extreme influence. Furthermore, the results of questionnaire were evaluated between two age groups (5–12 and 13–18 years).

An English translation of the full Italian questionnaire is available as **Supplementary Material**.

Demographic and clinical patients' characteristics and results of questionnaire were statistically analyzed. The numerical data were expressed as mean and standard deviation, and the categorical variables as absolute frequencies and percentages. In order to compare patients less or more than 12 years we applied Mann-Whitney test for numerical parameters and Chi Square test for categorical variables. The influence of patients' clinical and demographical characteristics (i.e., gender, age group, diabetes duration, type of insulin treatment and glucose monitoring system) on the perception of the impact of quarantine in the approach to T1D management was assessed by the cumulative proportional odds model. A *P*-value smaller than 0.050 was considered to be statistically significant.

TABLE 1 | Overview of the results of web-based survey.

N°	204
Age (years)	12.2 ± 3.6
Female (%)	86 (42.2%)
Duration of diabetes (years)	5.2 ± 3.7
Type of treatment	
Multiple daily injection	84 (41.2%)
Continuous subcutaneous insulin infusion	120 (58.8%)
Glucose monitoring system	
Self-monitoring blood glucose	56 (27.5%)
Continuous glucose monitoring or Flash glucose monitoring	148 (72.5%)
Modification of sleep-weak rhythm	
Yes	147 (72.5%)
No	55 (27.5%)
New skills acquired	
Cooking	54 (26.5%)
Reading books	28 (13.7%)
Do it yourself activities	35 (17.2%)
Art of music (singing, play an instrument)	18 (8.8%)
Housecleaning	12 (5.9%)
Others	49 (24%)
None	8 (3.9%)
Time spent on technology for recreational activities	
<1 h a day	36 (17.6%)
1–3 h a day	73 (35.8%)
4–6 h a day	43 (21.1%)
>6 h a day	44 (21.6%)
Not used	8 (3.9%)
Time spent on technology for educational purposes	
<1 h a day	14 (6.8%)
1–3 h a day	56 (27.5%)
4–6 h a day	69 (33.8%)
>6 h a day	59 (28.9%)
Not used	6 (2.9%)
Variations in eating habits	
Increased carbohydrate consumption	54 (26.5%)
Increased fat consumption	16 (7.8%)
Increased protein consumption	18 (8.8%)
No differences	116 (56.9%)
Time spent on physical activity at home	
<1 h a week	30 (14.7%)
1–3 h a week	57 (27.9%)
4–6 h a week	27 (13.2%)
>6 h a week	15 (7.4%)
Not practiced	75 (36.7%)
Variations in the approach to glucose monitoring	
More intensive	69 (33.8%)
Less intensive	38 (18.6%)
No differences	97 (47.5%)
How to contact the diabetes team	
Email messages	35 (17.2%)
Phone calls	18 (8.8%)

(Continued)

TABLE 1 | Continued

Text messages	51 (25%)
No contact	100 (49%)
How much the quarantine influenced the approach to the disease	
No influence	33 (16.2%)
Poor influence	78 (38.2%)
Relevant influence	74 (36.3%)
Extreme influence	19 (9.3%)

RESULTS

Mean age of our study population was 12.2 ± 3.6 years, with a prevalence of male gender (57.8%). Mean duration of T1D was 5.2 ± 3.7 years. Interestingly, most patients (72.5%) modified their sleep-wake rhythm. The use of technology was predominant both for recreational activities (communications, games, videos) and for educational purposes (scholar, musical and sportive activities). Less than 5% declared they did not use technology during this quarantine period. The average time spent on technology was mostly 4–6 h a day for educational purposes, and 1–3 h a day for recreational activities. Almost all the subjects (96.1%) took advantage of this period to acquire new skills, such as cooking, do it yourself activities learning, and reading books (13.7%). Regarding the dietary lifestyle, more than half of patients (56.9%) did not change their eating habits during the lock-down period. Fifty-four subjects (26.5%) increased carbohydrate consumption, 16 (7.8%) and 18 (8.8%) patients ate a large amount of fat and protein, respectively. Despite Italian governmental decrees prohibited outdoor sports, 63.3% of our patients regularly practiced physical activity at home. Particularly, 27.9% of patients spent from 1 to 3 h a week for physical activities, 14.7% of subjects spent less than an hour a week to do sports, and 20.6% of patients declared that physical activities kept them busy at least 4 h a week. Regarding the daily glucose monitoring, 33.8% of patients reported that it was more intensive during the quarantine period. Instead, 18.6% of the study participants paid less attention to their glycemic levels, and 47.5% of patients did not report differences from the pre-quarantine period. Interestingly, almost half of the patients (49%) did not need to contact the Diabetes team for advice on managing their disease. The most common used communication modality between patients and diabetes specialists was text messages, followed by e-mail messages and phone calls. None of the surveyed patients needed to be acutely evaluated during the lockdown period for diabetes-related acute complications (i.e., severe hypoglycemia, diabetic ketoacidosis). Finally, 45.6% of patients reported that the quarantine was an additional heavy burden on their perspective of the disease. Among these patients, 36.3% reported a relevant impact and 9.3% referred an extreme impact. On the contrary, 16.2% of the study participants declared that the quarantine did not affect their psychological and practical approach to diabetes, and 38.2% of patients partially suffered the consequence caused by the lock-down measures (**Table 1**).

TABLE 2 | Differences regarding the management of type 1 diabetes between patients <12 years group and patients ≥12 years group.

Variables	Patients ≤12 years	Patients >12 years	*P*-value
Gender			0.571
Male	55.9%	59.8%	
Female	44.1%	40.2%	
Duration of T1D (years)	3.8 ± 2.6	6.6 ± 4.1	**<0.001**
Type of treatment			0.155
Multiple daily injection	46.1%	36.3%	
Continuous subcutaneous insulin infusion	53.9%	63.7%	
Glucose monitoring system			0.120
Self-monitoring blood glucose	22.5%	32.4%	
Continuous glucose monitoring or flash glucose monitoring	77.5%	67.6%	
Variations in the approach to glucose monitoring			**0.028**
More intensive	39.2%	28.4%	
Less intensive	22.5%	14.7%	
No differences	38.2%	56.9%	
Time spent on physical activity at home			**0.001**
<1 h a week	18.6%	10.8%	
1–3 h a week	22.5%	33.3%	
4–6 h a week	7.8%	18.6%	
>6 h a week	3.9%	10.8%	
Not practiced	47.1%	26.5%	
Variations in eating habits			0.195
Increased carbohydrate consumption	21.5%	25.5%	
Increased fat consumption	8.8%	6.9%	
Increased protein consumption	4.9%	8.8%	
No differences	64.7%	58.8%	
How to contact the diabetes team			0.271
Email messages/Phone calls/Text messages	54.9%	47.1%	
No contact	45.1%	52.9%	
The influence of quarantine on the approach to diabetes			**0.017**
No influence	12.7%	19.6%	
Poor influence	31.4%	45.1%	
Relevant influence	42.2%	30.4%	
Extreme influence	13.7%	4.9%	

The bold values mean that the result is statistically significant.

When the two age groups were compared (**Table 2**), a significant difference was found in the duration of T1D

TABLE 3 | The relationship between patients' clinical and demographical characteristics and the perception of the influence of quarantine in their approach to type 1 diabetes.

Variables	Coefficient	95%C.I.	*P*-value
Constant 1*	−2.141	−2.949; −1.332	**<0.001**
Constant 2*	−0.260	−1.006; 0.485	0.494
Constant 3*	1.910	1.087; 2.733	**<0.001**
Gender	−0.162	−0.684; 0.360	0.543
Age group	−0.779	−1.343; −0.215	**0.007**
Diabetes duration	−0.005	−0.083; 0.072	0.898
Type of treatment	0.102	−0.482; 0.687	0.731
Glucose monitoring systems	0.025	−0.608; 0.658	0.938

**The cumulative proportional odds model uses a number of constants equal to the number of the variables included in the outcome (response levels regarding the influence of the lock-down period on the approach to the disease) minus 1.*
The bold values mean that the result is statistically significant.

($P < 0.001$). Older patients reported they spent more hours for physical activities than younger subjects ($P < 0.001$). On the contrary, patients aged ≤12 years measured glucose levels more frequently ($P = 0.028$). Interestingly, they were significantly more influenced by the quarantine period in their approach to the disease than older patients ($P = 0.017$). No further differences were found between the two groups. The cumulative proportional odds model showed that the younger age was the only factor that was significantly related to the different perception of the influence of quarantine in the approach to T1D management in our study population ($P = 0.007$) (**Table 3**).

DISCUSSION

T1D is a metabolic disease characterized by the progressive decline of pancreatic β cells functions leading to relative or absolute insulin deficiency (7). T1D is one of the most frequent autoimmune disorders in the pediatric population, and its incidence and prevalence are increasing worldwide (8). It is estimated that about 18,000 children and adolescents are currently affected by T1D in Italy (9). Evidence shows that a large number of pediatric patients, especially diabetic adolescents, experience disease-related impairment of quality of life and may develop depression, anxiety and other psychological states (10, 11). Psychological and behavioral disorders in T1D pediatric patients have been demonstrated to be related to negative health outcomes, such as brittle glycemic control and high risk of acute and chronic complications (12).

Although no data are available on the exact number of symptomatic and asymptomatic subjects positive for COVID-19 in the pediatric age, children appear to be less infected and if infected develop milder clinical pictures due to SARS-CoV-2 infection (13). A descriptive cases series of 130 Italian children with a confirmed diagnosis of COVID-19 reported that only 8.5% of these had a severe disease, and 6.9% had a critical presentation (14). Another Italian study involving a cohort of 100 hospitalized children affected by COVID-19 demonstrated that only nine patients needed respiratory support (15). Children and adolescents have been impacted psychologically experiencing various behavioral issues (6). The risk of acute stress disorder, adjustment disorder and grief in children who are quarantined during pandemic diseases had already been well-described (16). Furthermore, people suffering from a chronic disorder, such as T1D, are more vulnerable and at higher risk for developing dangerous feelings, such as uncertainties, distraction, irritability, and fear.

However, our results showed that most of the study participants reacted reassuringly to this new social condition as demonstrated by the responses regarding the management of the disease. In fact, the majority of children and adolescents with T1D were able to comply with the landmarks of the management of diabetes (i.e., healthy and balanced diet, regular physical activity and careful glucose monitoring). More than half of patients reported having avoided overeating during this quarantine period. We suppose that abstention from school and peer relationships out of school, has helped to maintain a healthy diet since numerous extra meals disappeared. Despite the lock-down measures, almost two third of our patients regularly have engaged in physical activity. Regular physical exercise is known to help subjects with T1D achieve good glycemic control, as well as improve lipid profile, body composition and well-being (17). Regarding the daily glucose monitoring, our results showed that the quarantine period negatively influenced only 18.6% of patients who reported a less intensive control of their glycemic values.

These findings suggest that pediatric patients with T1D developed functional "empowerment" as a response to the social emergency. Furthermore, they showed greater awareness of their disease and excellent coping skills by using technology in a proper way (18). Technology has played a crucial role in the quarantine approach for T1D children. In fact, technological medical devices (e.g., insulin pump, glucose sensor) have facilitated the management of the disease, while other technological tools, such as smartphones, tablets, and personal computers have preserved the "social dimension" even during the lock-down. Thanks to the availability of technology, children and adolescents have been able to continue school learning and to ensure social networks by minimizing negative emotions related to the social isolation.

Although physical freedom has been limited by the lockdown, new individual resources have been emerged due to personal and familiar factors, but also thanks to the school system and friendly system which have been kept active through technological tools (19).

Interestingly, patients >12 years reported having practiced indoor physical activities more regularly than younger patients and, above all, they were significantly less affected by the quarantine period in their approach to the disease. Adolescence is a well-known, high-risk time period for all young people who experience rebellion and lawlessness. It is widely demonstrated that the adolescent population with T1D is at high risk of poor clinical outcomes (20). However, our findings highlight that adolescence is also a crucial phase of life for the individual since it allows the achievement of a satisfactory level of interior maturity and new personal skills (21). Instead, patients ≤12 years were mainly affected by the quarantine as they are still in need for reassurance and parental care, and appear uncertain in the management of the disease because of recent diagnosis and/or poor autonomy. Therefore, our finding that this age group monitored glycemic levels more intensely could also be explained by the more rigorous parental control in the various aspects of T1D management.

In conclusion, the present study demonstrated that children and adolescents with T1D showed high levels of resilience. Although quarantine was a stressful psychological condition, pediatric patients were able to overcome their limits to reach new interior resources and strengthened self-awareness.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

FL conceived the designed study and approved the final version of the manuscript. SP drafted and wrote the paper. MP and FP analyzed the results and helped to write the paper. VD and PL sent the questionnaire link to the patients and collected the results. AA realized the statistical analysis. GP and GS contributed to the discussion and reviewed the paper. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fped.2020.00491/full#supplementary-material

REFERENCES

1. Yan Y, Shin WI, Pang YX, Meng Y, Lai J, You C, et al. The first 75 days of novel coronavirus (SARS-CoV-2) outbreak: recent advances, prevention, and treatment. *Int J Environ Res Public Health.* (2020) 17:2323. doi: 10.3390/ijerph17072323
2. Johns Hopkins Coronavirus Resource Center. *COVID-19 Map*. Johns Hopkins Coronavirus Resource Center. Available online at: https://coronavirus.jhu.edu/map.html (accessed July 1, 2020).
3. Buonsenso D, Onesimo R, Valentini P, Chiaretti A, Gatto A, Attinà G, et al. Children's healthcare during corona virus disease 19 pandemic: the Italian experience. *Pediatr Infect Dis J.* (2020) 39:e137–40. doi: 10.1097/INF.0000000000002732
4. Bressan S, Buonsenso D, Farrugia R, Parri N, Oostenbrink R, Titomanlio L, et al. Preparedness and response to pediatric CoVID-19 in European Emergency Departments: a survey of the REPEM and PERUKI networks. *Ann Emerg Med.* (2020). doi: 10.1016/j.annemergmed.2020.05.018. [Epub ahead of print].
5. Wang C, Pan R, Wan X, Tan Y, Xu L, Ho CS, et al. Immediate psychological responses and associated factors during the initial stage of the 2019 coronavirus disease (COVID-19) epidemic among the general population in China. *Int J Environ Res Public Health.* (2020) 17:1729. doi: 10.3390/ijerph17051729
6. Jiao WY, Wang LN, Liu J, Fang SF, Jiao FY, Pettoello-Mantovani M, et al. Behavioral and emotional disorders in children during the COVID-19 epidemic. *J Pediatr.* (2020) 221:264–6.e1. doi: 10.1016/j.jpeds.2020.03.013
7. American Diabetes Association. 2. Classification and diagnosis of diabetes: standards of medical care in diabetes-2020. *Diabetes Care.* (2020) 43:S14–31. doi: 10.2337/dc20-S002
8. Mobasseri M, Shirmohammadi M, Amiri T, Vahed N, Hosseini Fard H, Ghojazadeh M. Prevalence and incidence of type 1 diabetes in the world: a systematic review and meta-analysis. *Health Promot Perspect.* (2020) 10:98–115. doi: 10.34172/hpp.2020.18
9. Bruno G, Pagano E, Rossi E, Cataudella S, De Rosa M, Marchesini G, et al. Incidence, prevalence, costs and quality of care of type 1 diabetes in Italy, age 0–29 years: the population-based CINECA-SID ARNO Observatory, 2002–2012. *Nutr Metab Cardiovasc Dis.* (2016) 26:1104–11. doi: 10.1016/j.numecd.2016.09.002
10. Mommersteeg PMC, Herr R, Pouwer F, Holt RIG, Loerbroks A. The association between diabetes and an episode of depressive symptoms in the 2002 World Health Survey: an analysis of 231,797 individuals from 47 countries. *Diabet Med.* (2013) 30:e208–14. doi: 10.1111/dme.12193
11. Adal E, Önal Z, Ersen A, Yalçin K, Önal H, Aydin A. Recognizing the psychosocial aspects of type 1 diabetes in adolescents. *J Clin Res Pediatr Endocrinol.* (2015) 7:57–62. doi: 10.4274/jcrpe.1745
12. Stewart SM, Rao U, Emslie GJ, Klein D, White PC. Depressive symptoms predict hospitalization for adolescents with type 1 diabetes mellitus. *Pediatrics.* (2005) 115:1315–9. doi: 10.1542/peds.2004-1717
13. Passanisi S, Lombardo F, Salzano G, Pajno GB. Are children most of the submerged part of SARS-CoV-2 iceberg? *Front Pediatr.* (2020) 8:213. doi: 10.3389/fped.2020.00213
14. Parri N, Magistà AM, Marchetti F, Cantoni B, Arrighini A, Romanengo M, et al. Characteristic of COVID-19 infection in pediatric patients: early findings from two Italian Pediatric Research Networks. *Eur J Pediatr.* (2020) 179:1315–23. doi: 10.1007/s00431-020-03683-8

15. Parri N, Lenge M, Buonsenso D. Children with Covid-19 in Pediatric Emergency Departments in Italy. *N Engl J Med.* (2020) 383:187–90. doi: 10.1056/NEJMc2007617
16. Sprang G, Silman M. Posttraumatic stress disorder in parents and youth after health-related disasters. *Disaster Med Public Health Prep.* (2013) 7:105–10. doi: 10.1017/dmp.2013.22
17. Riddell MC, Gallen IW, Smart CE, Taplin CE, Adolfsson P, Lumb AN, et al. Exercise management in type 1 diabetes: a consensus statement. *Lancet Diabetes Endocrinol.* (2017) 5:377–90. doi: 10.1016/S2213-8587(17)30014-1
18. Funnell MM, Nwankwo R, Gillard ML, Anderson RM, Tang TS. Implementing an empowerment-based diabetes self-management education program. *Diabetes Educ.* (2005) 31:53, 55-6, 61. doi: 10.1177/0145721704273166
19. Goldschmidt K. The COVID-19 pandemic: technology use to support the wellbeing of children. *J Pediatr Nurs.* (2020) 53:88–90. doi: 10.1016/j.pedn.2020.04.013
20. Raymond J. Updates in behavioural and psychosocial literature in adolescents with type 1 diabetes. *Curr Opin Endocrinol Diabetes Obes.* (2015) 22:265–9. doi: 10.1097/MED.0000000000000167
21. Oosterhoff B, Wray-Lake L, Palmer CA, Kaplow JB. Historical trends in concerns about social issues across four decades among U.S. adolescents. *J Res Adolesc.* (2020) 30:485–98. doi: 10.1111/jora.12493

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling editor declared a past co-authorship with several of the authors FL and GS.

frontiers in Public Health

SYSTEMATIC REVIEW
published: 04 August 2020
doi: 10.3389/fpubh.2020.00432

D-Dimer Concentrations and COVID-19 Severity: A Systematic Review and Meta-Analysis

Panagiotis Paliogiannis[1], *Arduino Aleksander Mangoni*[2], *Paola Dettori*[3], *Gheyath K. Nasrallah*[4,5]*, *Gianfranco Pintus*[6,7]* and *Angelo Zinellu*[7]

[1] Department of Medical, Surgical and Experimental Sciences, University of Sassari, Sassari, Italy, [2] Department of Clinical Pharmacology, College of Medicine and Public Health, Flinders University, Adelaide, SA, Australia, [3] Cure and Health Center, Sassari, Italy, [4] Department of Biomedical Sciences, College of Health Sciences, Qatar University, Doha, Qatar, [5] Biomedical Research Centre, Qatar University, Doha, Qatar, [6] Department of Medical Laboratory Sciences, College of Health Sciences, and Sharjah Institute for Medical Research, University of Sharjah, Sharjah, United Arab Emirates, [7] Biomedical Sciences, University of Sassari, Sassari, Italy

OPEN ACCESS

Edited by:
Zhongheng Zhang,
Sir Run Run Shaw Hospital, China

Reviewed by:
Masoud Sadeghi,
Kermanshah University of Medical Sciences, Iran
Loïc Deblais,
The Ohio State University, United States
Lin Chen,
Jinhua Central Hospital, China
Takafira Mduluza,
University of Zimbabwe, Zimbabwe

***Correspondence:**
Gheyath K. Nasrallah
gheyath.nasrallah@qu.edu.qa
Gianfranco Pintus
gpintus@sharjah.ac.ae

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Public Health

Received: *26 May 2020*
Accepted: *14 July 2020*
Published: *04 August 2020*

Citation:
Paliogiannis P, Mangoni AA, Dettori P, Nasrallah GK, Pintus G and Zinellu A (2020) D-Dimer Concentrations and COVID-19 Severity: A Systematic Review and Meta-Analysis. Front. Public Health 8:432. doi: 10.3389/fpubh.2020.00432

Coronavirus disease 2019 (COVID-19) is a recently described infectious disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Since late 2019, COVID-19 has rapidly spread in virtually all countries, imposing the adoption of significant lockdown and social distancing measures. The activation of the coagulation cascade is a common feature of disseminated intravascular coagulation and adverse clinical outcomes in COVID-19 patients. In this study, we conducted a meta-analysis aiming to investigate differences in serum D-dimer concentrations in patients with and without severe COVID-19 disease. An electronic search in Medline (PubMed), Scopus and Web of Science was performed with no language restrictions, and 13 articles were reporting on 1,807 patients (585, 32.4% with severe disease) were finally identified and included in the meta-analysis. The pooled results of all studies revealed that the D-dimer concentrations were significantly higher in patients with more severe COVID-19 (SMD: 0.91 mg/L; 95% CI, 0.75 to 1.07 mg/L, $p < 0.0001$). The heterogeneity was moderate ($I^2 = 46.5\%$; $p = 0.033$). Sensitivity analysis showed that the effect size was not modified when any single study was in turn removed (effect size range, 0.87 mg/L to 0.93 mg/L). The Begg's ($p = 0.76$) and Egger's tests ($p = 0.38$) showed no publication bias. In conclusion, our systematic review and meta-analysis showed that serum D-dimer concentrations in patients with severe COVID-19 are significantly higher when compared to those with non-severe forms.

Keywords: D-dimer, coagulation, thrombosis, COVID-19, SARS-CoV-2

INTRODUCTION

Coronavirus disease 2019 (COVID-19) is a recently described infectious disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (1). Since late 2019, COVID-19 has rapidly spread in virtually all countries, affecting more than two million people and causing more than 150,000 deaths worldwide (data from 17 Apr 2020, https://www.worldometers.info/coronavirus/). These figures are continuously growing despite the

adoption of significant lockdown and social distancing measures, particularly in Eastern Asia, Europe, and North America (2). The rapid expansion and the relatively high lethality may depend on several biological characteristics, such as the high infectivity of SARS-CoV-2, the high percentage of asymptomatic vectors, and the relatively long incubation period (3). However, significant knowledge gaps remain in the pathophysiology of the disease. In this context, a better knowledge of the factors that are responsible for the development of significant clinical complications in a subgroup of COVID-19 patients, indicating high disease severity, might lead to the identification of better pharmacological and non-pharmacological therapies and care pathways. This would improve patient outcomes, and reduce the current burden on health care systems, pending the development of effective vaccines. There is increasing evidence that SARS-CoV-2 induces, in severe cases, a cytokine storm that triggers the coagulation cascade, causing thrombotic complications (4). This is clinically relevant as the activation of the coagulation cascade is a common feature of disseminated intravascular coagulation (DIC) and adverse clinical outcomes in COVID-19 patients and appears to be more frequent than what observed in patients suffering from severe forms of SARS-CoV in 2003 (5). The key pathophysiological role of DIC in the clinical progress of COVID-19 is further supported by the presence, in autopsies of patients succumbing to the disease, of fibrinous thrombi, endothelial tumefaction, and megakaryocytes in small pulmonary arteries and pulmonary capillaries (6).

The D-dimer, a fibrin degradation product, is a relatively small protein fragment that is present in the blood following degradation of blood clots by fibrinolysis. The determination of circulating D-dimer concentrations is a sensitive test in clinical practice to diagnose thrombotic states, including pulmonary embolism and DIC (7). Therefore, elevations in D-dimer levels in COVID-19 patients might be helpful to rapidly identify those that have high disease severity, pulmonary complications, and risk of venous thromboembolism in the setting of a pro-thrombotic state. This would assist with risk stratification and the early introduction of therapeutic measures that might reduce COVID-19 related morbidity and mortality.

A recent meta-analysis has shown that patients with severe forms of COVID-19 have higher D-dimer concentrations when compared to those with milder forms (8). However, only a small number of studies in a total of 553 patients were selected. Furthermore, in this meta-analysis the heterogeneity across the studies was extremely high, I^2 94%, $P < 0.001$). Therefore, we conducted an updated meta-analysis that takes into account additional studies to investigate differences in serum D-dimer concentrations in patients with and without severe COVID-19 disease.

MATERIALS AND METHODS

Study Search and Selection

An electronic search in Medline (PubMed interface), Scopus, and Web of Science was performed using the keywords "D-dimer" AND "coronavirus" OR "D-dimer" AND "COVID-19." The inclusion criteria were: (a) studies reporting continuous data on serum D-dimer concentrations in COVID-19 patients, (b) articles dividing COVID-19 patients in severity classes, (c) articles including adult patients, (d) studies approved by an ethical committee, and (e) articles published from 1st January 2020 to the date of the electronic search (14th April, 2020). There were no language restrictions. The titles, abstracts and full texts of the publications retrieved were screened by two independent investigators (PP and AZ). The reference list of the studies identified was also checked in order to identify additional studies. The Newcastle—Ottava Scale (NOS) was used for quality assesment. This meta-analysis was conducted according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (**Supplementary Materials**).

Statistical Analysis

Standardized mean differences (SMD) were used to build forest plots of continuous data and to evaluate differences in serum D-dimer concentrations between severe and non-severe patients with COVID-19 disease. A P-value < 0.05 was considered statistically significant, and 95% confidence intervals (CIs) were reported. When necessary, the mean and standard deviation values were extrapolated from median and IQR values, as previously reported by Wan et al. (9). Heterogeneity of SMD

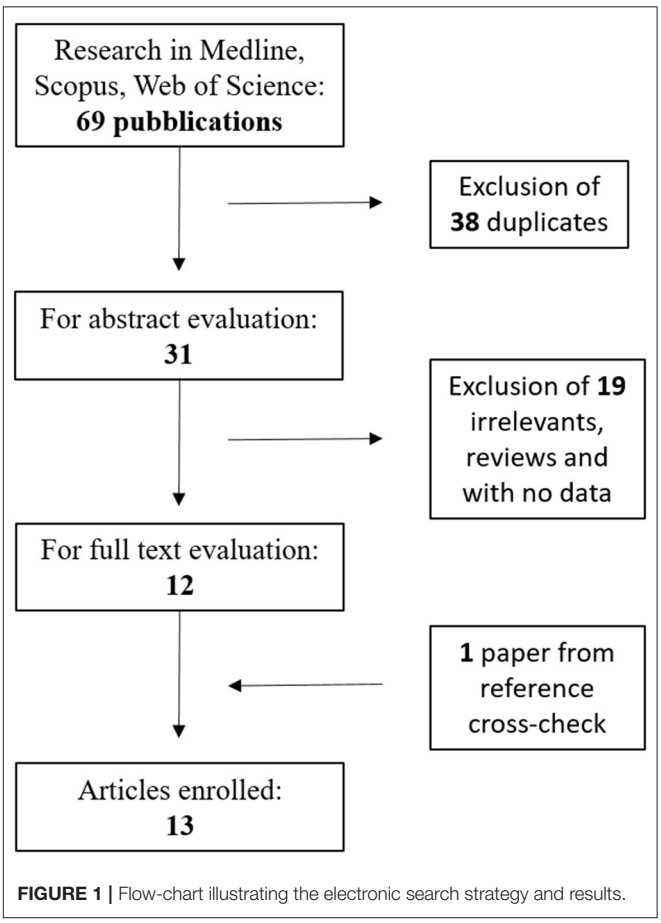


FIGURE 1 | Flow-chart illustrating the electronic search strategy and results.

TABLE 1 | Characteristics of the patients and D-dimer values in the studies enrolled for meta-analysis.

References	NOS stars	Total cases (severe)	Age	Males, *n* (%)	D-dimer total (mg/L)	D-dimer severe (mg/L)	D-dimer non-severe (mg/L)
Zhou et al. (16)	6	17 (5)	NA	6 (35)	NA	0.28 ± 0.11	0.29 ± 0.11
Tang et al. (17)	7	449 (134)	65 (mean)	268	1.94 (0.90–9.44)	4.70 (1.42–21.00)	1.47 (0.78–4.16)
Chen et al. (18)	6	21 (11)	56 (median)	17 (81)	0.5 (0.4–1.8)	2.6 (0.6–18.7)	0.3 (0.3–0.4)
Chen et al. (19)	6	274 (113)	62 (median)	171 (62)	1.1 (0.5–3.2)	4.6 (1.3–21.0)	0.6 (0.3–1.3)
Wan et al. (20)	6	135 (40)	47 (median)	72 (53)	0.4 (0.2–0.6)	0.6 (0.4–1.1)	0.3 (0.2–0.5)
Gao et al. (21)	6	43 (15)	45 (mean)	26 (58)	NA	0.49 (0.29-0.91)	0.21 (0.19–0.27)
Han et al. (22)	6	84 (35)	NA	NA	NA	19.11 ± 35.48	2.14 ± 2.88
Zhou et al. (23)	7	191 (54)	56 (median)	119 (62)	0.8 (0.4–3.2)	5.2 (1.5–21.1)	0.6 (0.3–1.0)
Wu et al. (24)	6	201 (84)	51 (median)	128 (64)	0.61 (0.35–1.28)	1.16 (0.46–5.37)	0.52 (0.33–0.93)
Liu et al. (25)	6	30 (4)	35 (mean)	10 (33)	NA	1.54 ± 1.22	0.26 ± 0.08
Zhang et al. (26)	7	138 (56)	57 (median)	71 (51)	0.2 (0.1–0.5)	0.4 (0.2–2.4)	0.2 (0.1–0.3)
Tang et al. (27)	7	183 (21)	54 (mean)	98 (53)	0.66 (0.38–1.50)	2.12 (0.77–5.27)	0.61 (0.35–1.29)
Huang et al. (28)	7	41 (13)	49 (median)	30 (73)	0.5 (0.3–1.3)	2.4 (0.6–14.4)	0.5 (0.3–0.8)
Total		1,807 (585)					

NOS, Newcastle – Ottawa Scale; NA, not available.

across studies was tested using the Q statistic (significance level at $p < 0.10$). The I^2 statistic, a quantitative measure of inconsistency across studies, was also calculated ($I^2 < 25\%$, no heterogeneity; I^2 between 25 and 50%, moderate heterogeneity; I^2 between 50 and 75%, large heterogeneity; and $I^2 > 75\%$, extreme heterogeneity) (10, 11). In analyses in which heterogeneity was high, a random-effects model was applied. To investigate the influence of an individual study on the overall risk estimate, a sensitivity analysis was conducted by sequentially excluding one study at a time (12). Begg's adjusted rank correlation test and Egger's regression asymmetry test, for the analysis of associations between study size and magnitude of effect were used to evaluate the presence of potential publication bias (13, 14). The Duval and Tweedie "trim and fill" procedure to identify and correct for funnel plot asymmetry arising from publication bias was also used (15). Statistical analyses were performed using Stata 14 (STATA Corp., College Station, TX, USA).

RESULTS

Study Selection Results and Characteristics

The flow diagram of the literature search performed is presented in **Figure 1**. From an initial total of 69 studies, 13 were finally identified and included in the meta-analysis (16–28); the total number of COVID-19 patients in these studies was 1,807. Among them, 585 (32.4%) were affected by a severe form of COVID-19 (**Table 1**). The NOS quality assessment is described in **Table 1**.

All selected studies were conducted in China. Six articles defined severe cases based on current clinical guidelines (20–22, 24–26), four defined severe cases as those who died in comparison to survivors (17, 19, 23, 27), and three had alternative definitions (disease progression vs. no progression or admission vs. no admission to intensive care units) (16, 18, 28).

Meta-Analysis

The mean differences in serum D-dimer concentrations between COVID-19 patients with or without severe disease in the 13 studies are shown in **Figure 2**. In 12 studies, patients with severe COVID-19 displayed higher D-dimer serum concentrations when compared to those with milder forms (mean difference range, 0.62–3.15 mg/L) (17–28). By contrast, in the remaining study, the D-dimer concentration was found to be mildly higher in patients with non-severe forms of COVID-19 (mean difference 0.09 mg/L) (16). The pooled results of all studies revealed that the D-dimer concentrations were significantly higher in patients with more severe COVID-19 (SMD: 0.91 mg/L; 95% CI, 0.75 to 1.07 mg/L, $p < 0.0001$). The heterogeneity was moderate ($I^2 = 46.5\%$; $p = 0.033$). Sensitivity analysis showed that the effect size was not modified when any single study was in turn removed (effect size range, 0.87 mg/L−0.93 mg/L, **Figure 3**). The Begg's ($p = 0.76$) and Egger's tests ($p = 0.38$) showed no publication bias. Accordingly, the trim-and-fill analysis found that no study was missing or should be added (**Figure 4**). In meta-regression analysis, no correlation was found either between SMD and age ($p = 0.37$) or between SMD and gender ($p = 0.41$). Notably, the age ratio between patients with more severe COVID-19 and those with milder forms was relatively small, between 1.0 and 1.3, in all studies. In addition, as reported in **Figure 5**, there were no significant differences in SMD values between the subgroup of patients classified according to guidelines (SMD: 0.94 mg/L; 95% CI 0.78 to 1.10 mg/L, $p < 0.0001$) and the subgroup classified as dead or survivors (SMD: 0.97 mg/L; 95% CI 0.65 to 1.29 mg/L, $p < 0.0001$), although in the first group a significantly lower heterogeneity was observed ($I^2 = 21.4\%$, $p < 0.28$ vs. $I^2 = 65.5\%$, $p < 0.013$).

DISCUSSION

Our updated meta-analysis of 13 studies in 1,807 COVID-19 patients showed that the serum D-dimer concentrations in

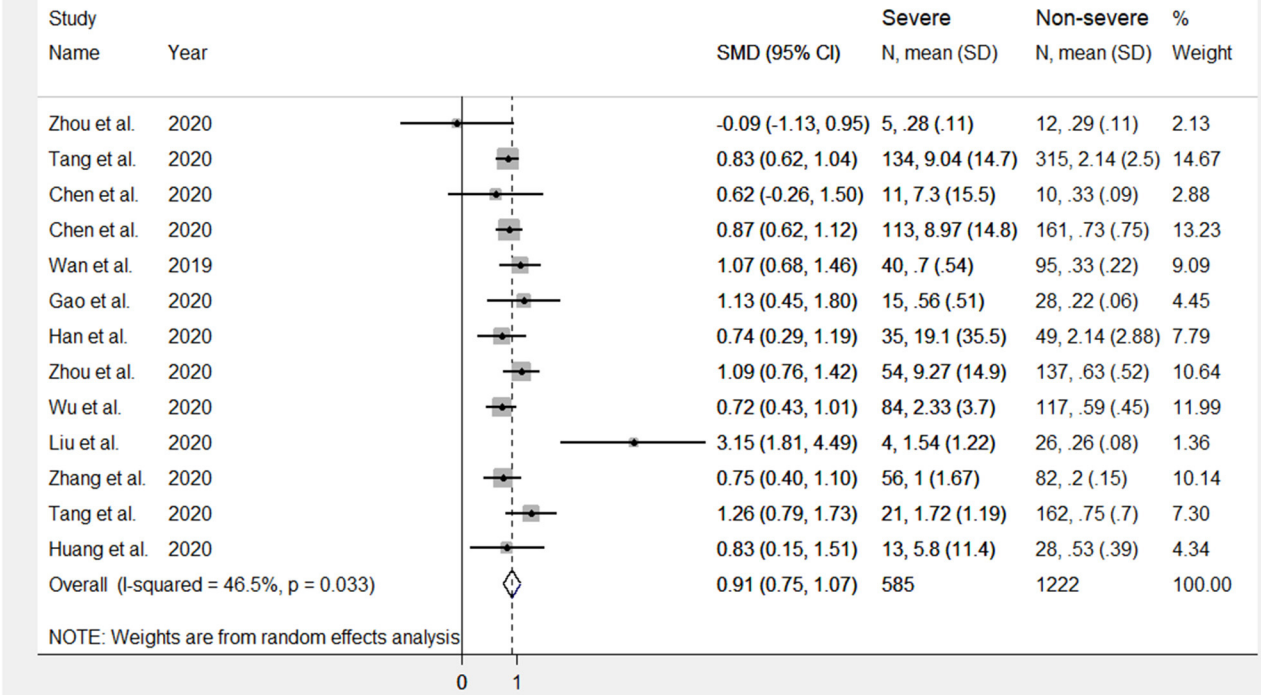


FIGURE 2 | Forest plot illustrating D-dimer standardized mean differences (SMD) in patients with and without severe COVID-19.

patients with severe forms of the disease were significantly higher than those in patients with milder forms. When compared to a recent meta-analysis of four studies in a total of 553 COVID-19 patients, the observed SMD values were relatively small, 0.91 mg/L (3, 17, 27, 28). Furthermore, in our meta-analysis the heterogeneity was substantially lower, I^2 46.5 vs. 94% (8). These results further support the presence of a pro-thrombotic state, and possibly DIC, in COVID-19 patients with severe disease, potentially accounting for the structural and functional lung abnormalities commonly reported in this subgroup. In support of this hypothesis, recent autoptic reports have shown alterations compatible with DIC in the lungs of COVID-19 patients (6). Interestingly, we observed no significant associations between increasing SMD values and the age ratio between patients with more severe COVID-19 and those with milder forms, despite the established age-related increase in serum D-dimer concentrations (7). As patients with severe COVID-19 disease are also significantly older than subgroups with milder forms (29), our findings suggest that the reported differences in serum D-dimer concentrations are independent of age differences in patients with different disease severity. Although this further supports the presence of DIC as the primary marker of D-dimer elevations and COVID-19 severity, additional studies in cohorts with higher age ratios between patients with more severe COVID-19 and those with milder forms are required to confirm this proposition.

Pending further research to investigate the cause-effect relationship between serum D-dimer concentrations, COVID-19 disease severity, the onset of pulmonary complications and clinical outcomes, the identification of D-dimer as a biomarker of COVID-19 severity is potentially clinically relevant. Its relatively simple and inexpensive determination might assist, particularly with serial assessments, with the rapid identification of those patients developing DIC, pulmonary compromise, or at risk of venous thromboembolism that requires aggressive care and intensive monitoring (30). While the development of COVID-19 vaccines is eagerly awaited, a better understanding of the pathophysiological mechanisms responsible for the clinical deterioration and increased risk of death in affected patients is likely to be beneficial. For example, the rapid initiation of DIC therapies, instigated by high D-dimer concentrations and the presence of other diagnostic criteria, might provide additional therapeutic advantages in severe COVID-19 patients already receiving ventilatory and circulatory support (30). This proposition is supported by the findings of a recent study in 449 severe COVID-19 patients with significant elevations of serum D-dimer concentrations and/or criteria for DIC. The administration of low molecular weight heparin in these patients was associated with a significant improvement in 28-day survival when compared to non-users (27).

The moderate heterogeneity in the studies enrolled might depend on the different definitions of disease severity; in six studies, available clinical guidelines were followed, mainly

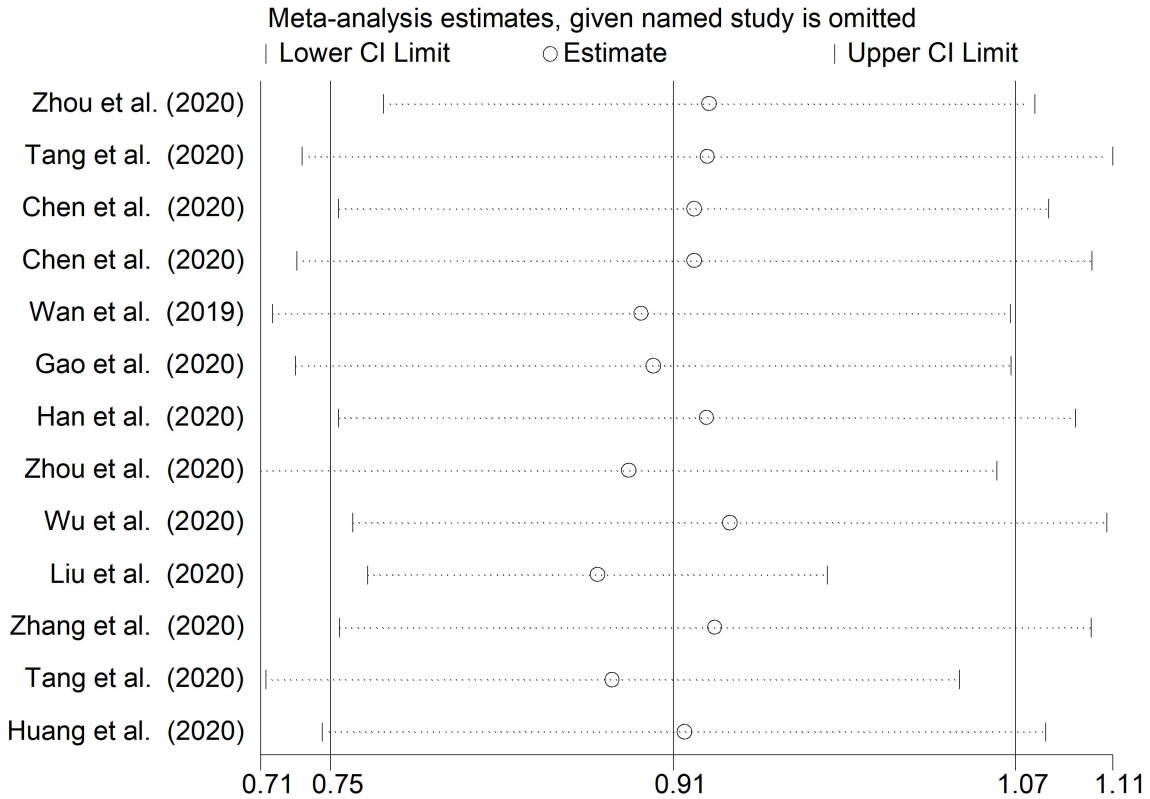


FIGURE 3 | Sensitivity analysis of the studies enrolled. CI, confidence interval.

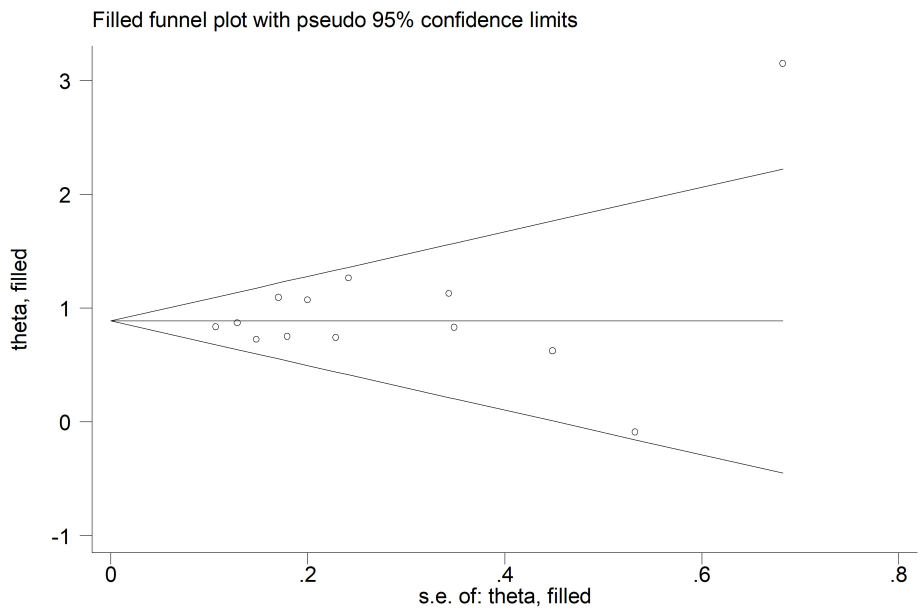


FIGURE 4 | Trim-and-fill analysis of the studies enrolled.

the "new coronavirus pneumonia diagnosis and treatment plan" (versions 4 and 5) developed by the National Health Committee of the People's Republic of China (31). In four studies, the severity of the disease was based on survivorship or death, and finally, in the remaining three studies, further classifications were used, such as disease progression vs. no

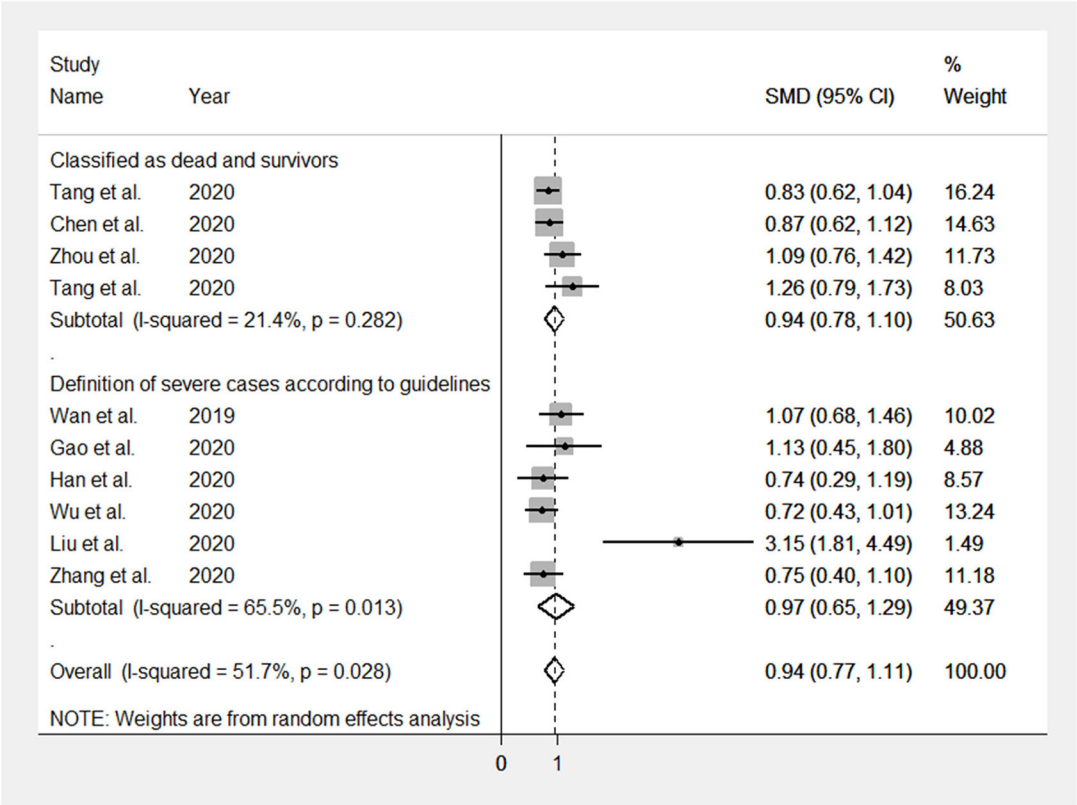


FIGURE 5 | Forest plots illustrating subgroup analyses on the basis of the definition of COVID-19 severity. SMD: standardized mean difference.

progression, and admission or no admission in intensive care units. For this reason, we performed subgroup analyses, which showed no significant differences in SMD values between the subgroup of patients classified according to clinical guidelines and the subgroup classified as dead or survivors. Further potential issues are that all the included studies were carried out in China, no strict diagnostic performance was investigated, and no specific guidelines for reporting (such as the Standards for Reporting Diagnostic Accuracy Studies, STARD recommendations) were followed in each individual study. Other potential sources of heterogeneity, not described in the identified studies, include differences in the timing of blood sample collection and analytical protocols for D-dimer measurement.

In conclusion, our systematic review and meta-analysis showed that the serum concentrations of D-dimer, a fibrin degradation product that is used to diagnose the presence of a pro-thrombotic state, are significantly higher in patients with severe COVID-19 when compared to those with non-severe forms. This suggests that D-dimer concentrations might be helpful to rapidly identify COVID-19 patients with high risk of pulmonary complications and venous thromboembolism, facilitating the early initiation of effective therapies. However, further studies are required to confirm such findings in different geographical areas, using robust assessment methods, and to investigate the associations between D-dimer concentrations, COVID-19 disease progress, response to treatment, and overall clinical prognosis.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

Conceptualization: AZ. Methodology: PP and AM. Software and writing—original draft preparation: PP, AM, and PD. Validation: PP, AM, and AZ. Formal analysis: PP, PD, GN, and GP. Investigation and resources: AZ, PP, AM, GN, and GP. Data curation and project administration: GN and GP. Writing—review and editing and funding acquisition: GN, GP, and AZ. Visualization: PP and AM. Supervision: AZ. All authors have read and agreed to the published version of the manuscript.

FUNDING

This research was funded by Qatar University [IRCC-2019-007] to GN and GP, Regione Autonoma della Sardegna [RASSR82005] to GP and AZ, and fondo UNISS di Ateneo per la ricerca 2019 to GP and AZ.

ACKNOWLEDGMENTS

The authors would like to thank Qatar National Library for sponsoring the publication fees of this paper.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2020.00432/full#supplementary-material

REFERENCES

1. Du Toit A. Outbreak of a novel coronavirus. *Nat Rev Microbiol.* (2020) 18:123. doi: 10.1038/s41579-020-0332-0
2. Rothan HA, Byrareddy SN. The epidemiology and pathogenesis of coronavirus disease (COVID-19) outbreak. *J Autoimmun.* (2020) 109:102433. doi: 10.1016/j.jaut.2020.102433
3. Wang W, Tang J, Wei F. Updated understanding of the outbreak of 2019 novel coronavirus (2019-nCoV) in Wuhan, China. *J Med Virol.* (2020) 92:441–7. doi: 10.1002/jmv.25689
4. Liu B, Li M, Zhou Z, Guan X, Xiang YJ. Can we use interleukin-6 (IL-6) blockade for coronavirus disease (2019). (COVID-19)-induced cytokine release syndrome (CRS)? *Autoimmun*. (2020) 111:102452. doi: 10.1016/j.jaut.2020.102452
5. Wong RS, Wu A, To KF, Lee N, Lam CW, Wong CK, et al. Haematological manifestations in patients with severe acute respiratory syndrome: retrospective analysis. *BMJ*. (2003) 326:1358–62. doi: 10.1136/bmj.326.7403.1358
6. Dolhnikoff M, Duarte-Neto AN, de Almeida Monteiro RA, Ferraz da Silva LF, Pierre de Oliveira E, Nascimento Saldiva PH, et al. Pathological evidence of pulmonary thrombotic phenomena in severe COVID-19. *J Thromb Haemost*. (2020) 18:1517–9. doi: 10.1111/jth.14844
7. Olson JD. D-dimer: an overview of hemostasis and fibrinolysis, assays, clinical applications. *Adv Clin Chem.* (2015) 69:1–46. doi: 10.1016/bs.acc.2014.12.001
8. Lippi G, Favaloro EJ. D-dimer is associated with severity of coronavirus disease 2019: A pooled analysis. *Thromb Haemost*. (2020) 120:876–8. doi: 10.1055/s-0040-1709650
9. Wan X, Wang W, Liu J, Tong T. Estimating the sample mean and standard deviation from the sample size, median, range and/or interquartile range. *BMC Med Res Methodol.* (2014) 14:135. doi: 10.1186/1471-2288-14-135
10. Bowden J, Tierney JF, Copas AJ, Burdett S. Quantifying, displaying and accounting for heterogeneity in the meta-analysis of RCTs using standard and generalised Q statistics. *BMC Med Res Methodol.* (2011) 11:41. doi: 10.1186/1471-2288-11-41
11. Higgins JP, Thompson SG. Quantifying heterogeneity in a meta-analysis. *Stat Med.* (2002) 21:1539–58. doi: 10.1002/sim.1186
12. Tobias A. Assessing the influence of a single study in the meta-analysis estimate. *Stat Tech Bull.* (1999) 47:15–7.
13. Begg CB, Mazumdar M. Operating characteristics of a rank correlation test for publication bias. *Biometrics.* (1994) 50:1088–101. doi: 10.2307/2533446
14. Sterne JA, Egger M. Funnel plots for detecting bias in meta-analysis: guidelines on choice of axis. *J Clin Epidemiol.* (2001) 54:1046–55. doi: 10.1016/S0895-4356(01)00377-8
15. Duval S, Tweedie R, Trim and Fill: A simple funnel-plot-based method of testing and adjusting for publication bias in meta-analysis. *Biometrics.* (2000) 56:455–63. doi: 10.1111/j.0006-341X.2000.00455.x
16. Zhou Y, Zhang Z, Tian J, Xiong S. Risk factors associated with disease progression in a cohort of patients infected with the 2019 novel coronavirus. *Ann Palliat Med.* (2020) 9:428–36. doi: 10.21037/apm.2020.03.26
17. Tang N, Bai H, Chen X, Gong J, Li D, Sun Z. Anticoagulant treatment is associated with decreased mortality in severe coronavirus disease 2019 patients with coagulopathy. *J Thromb Haemost.* (2020) 18:1094–9. doi: 10.1111/jth.14817
18. Chen G, Wu D, Guo W, Cao Y, Huang D, Wang H, et al. Clinical and immunological features of severe and moderate coronavirus disease 2019. *J Clin Invest.* (2020) 130:2620–9. doi: 10.1172/JCI137244
19. Chen T, Wu D, Chen H, Yan W, Yang D, Chen G, et al. Clinical characteristics of 113 deceased patients with coronavirus disease 2019: retrospective study. *BMJ.* (2020) 368:m1091. doi: 10.1136/bmj.m1091
20. Wan S, Xiang Y, Fang W, Zheng Y, Li B, Hu Y, et al. Clinical features and treatment of COVID-19 patients in northeast Chongqing. *J Med Virol.* (2020) 92:797–806. doi: 10.1002/jmv.25783
21. Gao Y, Li T, Han M, Li X, Wu D, Xu Y, et al. Diagnostic utility of clinical laboratory data determinations for patients with the severe COVID-19. *J Med Virol*. (2020) 92:791–6. doi: 10.1002/jmv.25770
22. Han H, Yang L, Liu R, Liu F, Wu KL, Li J, et al. Prominent changes in blood coagulation of patients with SARS-CoV-2 infection. *Clin Chem Lab Med*. (2020) 58:1116–20. doi: 10.1515/cclm-2020-0188
23. Zhou F, Yu T, Du R, Fan G, Liu Y, Liu Z, et al. Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: a retrospective cohort study. *Lancet*. (2020) 395:1054–62. doi: 10.1016/S0140-6736(20)30566-3
24. Wu C, Chen X, Cai Y, Xia J, Zhou X, Xu S, et al. Risk factors associated with acute respiratory distress syndrome and death in patients with coronavirus disease 2019 pneumonia in Wuhan, China. *JAMA Intern Med.* (2020) 180:1–11. doi: 10.1001/jamainternmed.2020.0994
25. Liu M, He P, Liu HG, Wang XJ, Li FJ, Chen S, et al. Clinical characteristics of 30 medical workers infected with new coronavirus pneumonia. *Zhonghua Jie He He Hu Xi Za Zhi.* (2020) 43:209–14. doi: 10.3760/cma.j.issn.1001-0939.2020.03.014
26. Zhang JJ, Dong X, Cao YY, Yuan YD, Yang YB, Yan YQ, et al. Clinical characteristics of 140 patients infected with SARS-CoV-2 in Wuhan, China. *Allergy*. (2020) 75:14238. doi: 10.1111/all.14238
27. Tang N, Li D, Wang X, Sun Z. Abnormal coagulation parameters are associated with poor prognosis in patients with novel coronavirus pneumonia. *J Thromb Haemost.* (2020) 18:844–7. doi: 10.1111/jth.14768
28. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet*. (2020) 395:497–506. doi: 10.1016/S0140-6736(20)30183-5
29. Chen T, Dai Z, Mo P, Li X, Ma Z, Song S, et al. Clinical characteristics and outcomes of older patients with coronavirus disease 2019. (COVID-19) in Wuhan, China (2019): a single-centered, retrospective study. *J Gerontol A Biol Sci Med Sci.* (in press) glaa089. doi: 10.1093/gerona/glaa089
30. Terpos E, Ntanasis-Stathopoulos I, Elalamy I, Kastritis E, Sergentanis TN, Politou M, et al. Hematological findings and complications of COVID-19. *Am J Hematol.* (2020) 95:834–47. doi: 10.1002/ajh.25829
31. *National Health Commission of the People's Republic of China*. Available online: http://www.nhc.gov.cn/ (accessed April 18, 2020).

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Medicine

ORIGINAL RESEARCH
published: 04 August 2020
doi: 10.3389/fmed.2020.00466

Ruxolitinib Rapidly Reduces Acute Respiratory Distress Syndrome in COVID-19 Disease. Analysis of Data Collection From RESPIRE Protocol

*Enrico Capochiani[1], Bruno Frediani[2], Giorgio Iervasi[3], Aldo Paolicchi[4], Spartaco Sani[5], Paolo Roncucci[6], Annarosa Cuccaro[1], Federico Franchi[7], Federico Simonetti[1], Davide Carrara[8], Ilaria Bertaggia[1], Daniela Nasso[1], Rossella Riccioni[1], Sabino Scolletta[7], Serafina Valente[9], Edoardo Conticini[2], Alessandro Gozzetti[10] and Monica Bocchia[10]**

[1] Hematology Unit, Center for Translational Medicine, Azienda USL Toscana NordOvest, Livorno, Italy, [2] Rheumatology Unit, COVID Unit, University of Siena, Azienda Ospedaliero Universitaria Senese, Siena, Italy, [3] Italian National Research Council (CNR) – Institute of Clinical Physiology (IFC), Pisa, Italy, [4] Clinical Pathology Unit, University of Pisa, Azienda Ospedaliero Universitaria Pisana, Pisa, Italy, [5] Infectious Disease Unit, COVID Unit, Azienda USL Toscana NordOvest, Livorno, Italy, [6] Critical Care Unit, COVID Unit, Azienda USL Toscana NordOvest, Livorno, Italy, [7] Anesthesia and Intensive Care Unit, COVID Unit, University of Siena, Azienda Ospedaliero Universitaria Senese, Siena, Italy, [8] Internal Medicine Unit, COVID, Unit Azienda USL Toscana NordOvest, Viareggio, Italy, [9] Cardiology Unit, COVID Unit, Azienda Ospedaliero Universitaria Senese, Siena, Italy, [10] Hematology Unit, Università of Siena, Azienda Ospedaliero Universitaria Senese, Siena, Italy

OPEN ACCESS

Edited by:
Zhongheng Zhang,
Sir Run Run Shaw Hospital, China

Reviewed by:
Mohamed Chakroun,
Hôpital Universitaire Fattouma Bourguiba, Tunisia
Pedro Xavier-Elsas,
Federal University of Rio de Janeiro, Brazil

***Correspondence:**
Monica Bocchia
bocchia@unisi.it

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: *24 May 2020*
Accepted: *13 July 2020*
Published: *04 August 2020*

Citation:
Capochiani E, Frediani B, Iervasi G, Paolicchi A, Sani S, Roncucci P, Cuccaro A, Franchi F, Simonetti F, Carrara D, Bertaggia I, Nasso D, Riccioni R, Scolletta S, Valente S, Conticini E, Gozzetti A and Bocchia M (2020) Ruxolitinib Rapidly Reduces Acute Respiratory Distress Syndrome in COVID-19 Disease. Analysis of Data Collection From RESPIRE Protocol. Front. Med. 7:466. doi: 10.3389/fmed.2020.00466

Background: The Coronavirus disease (COVID-19) pandemic is causing millions of infections and hundreds of thousands of deaths worldwide. Cumulative clinical and laboratory evidence suggest that a subset of patients with severe COVID-19 may develop a cytokine storm syndrome during the course of the disease, with severe respiratory impairment requiring ventilatory support. One field of research nowadays is to identify and treat viral-induced hyperinflammation with drugs used in other clinical conditions characterized by an hyperinflammation status. These drugs might help to reduce COVID19 mortality.

Methods: Ruxolitinib, a JAK1 and JAK2 inhibitor, has been successfully used to treat severe immune-mediated diseases, such as graft vs. host disease and Hemophagocytic lymphohistiocytosis. We used ruxolitinib in 18 patients with clinically progressive COVID-19 related acute respiratory distress syndrome, with a primary endpoint to rapidly reduce the degree of respiratory impairment and as a secondary endpoint to rapidly restore the PaO_2/FiO_2 ratio, as an evaluation of clinical status, and monitoring of drug related Adverse Events. Parameters of inflammation responses and organ functions were assessed and monitored. The treatment plan was ruxolitinib 20 mg bid for the first 48 h and subsequent two-step de-escalation at 10 mg bid and 5 mg bid for a maximum of 14 days of treatment.

Results: Our data collection shows a rapid clinical response with no evolution from *non-invasive ventilation* to mechanical ventilation in 16/18 patients and no response in two patients (overall response rate—ORR 89%). Already after 48 h of ruxolitinib treatment 16/18 patients showed evident clinical improvement, and after 7 days of treatment 11/18 patients showed fully recovered respiratory function (pO_2 > 98% in

spontaneous breathing), 4/18 patients had minimal oxygen requirement (2–4 L/m), 1/18 patient showed stable disease, and 2/18 patient showed progressive disease. After 14 days, 16/18 patients showed complete recovery of respiratory function (ORR 89%). Compliance to ruxolitinib planned treatment was 100% and no serious adverse event was recorded. In our case series of 18 critically ill patients with COVID-19 and ARDS, administration of ruxolitinib resulted in a clinical improvement that concurred to modify the standard course of disease. Ruxolitinib can be a therapeutic option for patients with respiratory insufficiency in COVID-19 related ARDS. RESPIRE Study (**R**uxolitinib for the treatment of acute r**ESPI**ratory dist**RE**ss syndrome, ClinicalTrials.gov Identifier: NCT04361903).

Keywords: COVID-19, ruxolitinib, respiratory distress syndrome, ICU, treatment

INTRODUCTION

Since December 2019, coronavirus disease (COVID-19) has led to a pandemic condition, requiring unprecedented public health interventions (1). From December 2019 up to date, millions of people have been infected and hundreds of thousands have died. The general mortality is about 1–5% in all COVID-19 cases, and the incidence of critical COVID-19, including both severe and life-threatening clinical pictures, is about 10–20%, with a much higher mortality rate (30–60%) (2).

Acute respiratory distress syndrome (ARDS), characterized by refractory hypoxemia and multi-organ dysfunction syndrome, is the leading cause of mortality in COVID-19 patients, placing a sudden and heavy burden on health care services (3, 4). It is currently believed that SARS-CoV-2 primarily infects the lungs, and subsequently causes systemic inflammation and immune response disorder, ultimately leading to multiple organ injury and even death (5). The available clinical treatment strategies to critical COVID-19 are mainly antiviral and oxygen therapy, as well as organ and symptomatic support, including mechanical ventilation, and even extracorporeal membrane oxygenation (ECMO) of cardiopulmonary support (6). However, the clinical efficacy of these strategies is still uncertain and the mortality rate of critical COVID-19 patients, as reported in clinical data from intensive-care units (ICUs), remains elevated (7). While efforts are focused on the development of safe and effective antivirals and vaccines, a growing body of evidence support the notion of an inflammatory excess, with cytokine upregulation, in patients with human coronavirus infections, including COVID-19, (8, 9) who develop severe respiratory impairment. Lung pathology (10) showed capillary leakage and recruitment of inflammatory cells, both from the adaptive and innate immune system, suggesting that adhesion molecules, chemokines, and the vascular endothelium are likely involved.

Cytokines' derangement in the context of COVID-19 resembles that of secondary hemophagocytic lymphohistiocytosis (sHLH), (11) which may be, indeed, triggered by viral infections. Both conditions share notable clinical features, such as fever and lung involvement, and both show increased levels of several cytokines, including interleukin (IL)-2, IL-6, IL-7, IL-10, Interferon alpha (IFN), tumor necrosis factor alpha (TNF-alfa), and chemotactic proteins (5). Some sHLH markers such as ferritin and IL-6 were found to be predictive of patients' outcome, thus suggesting a link between COVID-19 severity and the secondary inflammatory state. Moreover, evolution to ARDS is less likely in immunocompromised patients (12), especially in patients being treated with biological inhibitors or JAK inhibitors (13).

Anti-inflammatory agents were proposed (14) as reasonable options to counteract the overexuberant inflammatory response, with the aim of reducing mortality and rates of admission to ICU.

Ruxolitinib, a JAK1 and JAK2 inhibitor, is widely used for the treatment of myeloproliferative neoplasms, but has been successfully used to also treat immune-mediated diseases, such as graft vs. host disease (GvHD) (15, 16) and HLH (17), based on its rapid, potent, and pleiotropic influence on the host immune system. Based on these considerations, we hypothesized that

TABLE 1 | Inclusion and exclusion criteria.

Inclusion criteria
Positive analysis by real-time reverse transcriptase-polymer chain reaction [RT PCR (Shanghai BioTec or Sansure Biotech)] for SARS-CoV-2 of pharyngeal and nasal swabs
Non-pregnant male or female sex, aged 18 and over imaging [thoracic ultrasound, *chest X Ray (CXR) or* computed axial tomography (*CT scan)* positive for pneumonia]
Oxygen saturation (SaO_2) of 93% or less in on room air
Ratio of partial oxygen pressure (PaO_2) to inspired oxygen fraction (FiO_2) (PaO_2/FiO_2) less than 200 mg/Hg but not less than 100
Rapid clinical evolution with worsening respiratory parameters in the last 12 h
Exclusion criteria
Known hypersensitivity to the drugs
Patients in assisted breathing with tracheal cannula
Patients with active and undetailed serious illnesses prior to COVID-19 infection
Patients with kidney failure
Patients with positive Quantiferon TB test
Patients with unchecked documented bacterial or fungal sepsis (excluding procalcitonin in the presence of negative hemocultures)
Patients with neutropenia of 1,000 neutrophils/μl or less
Patients with platelets of 100,000/μl or less

immune-modulation with ruxolitinib might have been beneficial in reducing severity of ARDS in the context of COVID-19.

Here, we present the results of the retrospective multicenter observational study RESPIRE.

METHODS

Study Design and Participants

This multicenter retrospective cohort study was performed in three hospitals designated by Tuscany Regional Health Service Administration as treatment centers for COVID-19 (Livorno, Viareggio, Siena). The data collection period was from 10 March 2020 and the data cutoff date was 7 April 2020. Inclusion and exclusion criteria are summarized in **Table 1**.

Procedures

Ruxolitinib was used as off-label therapy, in patients with COVID-19 related ARDS. All patients were treated after written informed consent was provided. Informed consent was in accordance with General Data Protection Regulation (GDPR) (EU) 2016/679 and Italian Law 1998/94. The study was approved by the Italian COVID-19 Ethical Committee (National Institute for Infectious Diseases "Lazzaro Spallanzani") (trial register no. 81 April 2020). The data collection form included demographic, clinical, treatment, laboratory data, and prognosis. Detailed clinical data before and during ruxolitinib treatment were collected and obtained from the patient's electronic medical records. Other treatments delivered to the patients according to local guidelines for COVID-19, (e.g., azithromycin, heparin, steroids, etc.) have been preserved. The treatment plan included ruxolitinib 20 mg bid [same dose used in the hemophagocytic syndrome (17)] for the first 48 h and subsequent two-step de-escalation at 10 mg bids and 5 mg bids according to response achievement for a maximum total of 14 days of treatment.

In case of worsening of the respiratory status during the first 48 h, a reduction of dosage to 10 mg for the next 24 h and subsequent suspension of treatment was carried out.

The following data on the cohort of patients treated with ruxolitinib were retrospectively evaluated: the number of patients who had worsened respiratory function and from NIV needed MV; the time to restore PaO_2/FiO_2; compliance to the treatment and drug related AE, and overall survival as described in Respire Protocol.

For each patient treated with ruxolitinib, parameters of inflammation and organ function were measured before treatment (T0) and every 12, 24, or 48 h: vital parameters and respiratory function were monitored every 12 h and in any case in the presence of significant clinical changes. Serum cytokines: Interleukin 6 (IL6), T*umor Necrosis Factor* alpha (*TNF*-a), and Monocyte chemoattractant protein 1 (MCP-1), were measured every 48 h. Chest imaging was done as follows: T0: *chest X Ray (CXR)* and *thoracic ultrasound (TUS)*. In patients with deteriorating respiratory function a computed axial tomography (CT scan) was performed. CXR and US were the imaging technique used as follow up (bed-side) every 48 h. Routine blood chemistry examinations were performed every 24 h. All patients treated with ruxolitinib were admitted to the Intensive Care Unit (ICU) designated as the COVID-ICU and the decision to stop NIV and initiate MV was dependent on the ICU Medical Staff (18). We also retrospectively analyzed the outcomes (evolution from NIV to mechanical ventilation and life status) of all patients admitted in our COVID-ICU during the same period of time (March 2020 to April 2020) who did not receive ruxolitinib but who were treated according to the internal COVID-ICU Hospital guidelines.

Statistical Analysis

The categorical data were summarized as numbers and percentages, and inter-group comparisons were performed using Fisher's exact test. Continuous variables were expressed as the arithmetic mean and standard deviation (SD) or as the median and interquartile range, depending on whether or not they showed a gaussian distribution. Continuous data with gaussian distribution were compared with the Student's *t*-test or one-way ANOVA. Statistical analysis was performed using the SPSS Windows version 11.0 statistical package (SPSS Inc,

TABLE 2 | Demographics and clinical characteristics at baseline.

Age, Years (median)	62.5 (28.0–86.0)
Sex Male	12 (67%)
Female	6 (33%)
Comorbidity pts N (%)	6 (33%)
Comorbidity numbers	10
Hypertension	3 (2 pts)
Coronary heart disease	2 (2 pts)
Arrhythmia	2 (2 pts)
Diabetes	1 (1 pt)
Chronic obstructive lung disease	1 (1 pt)
Neoplasm	1 (1 pt)
Temperature (°C), median (IQR)	37.8 (37.1–39.3)
Pulse (beats per min), median (IQR)	89 (74–118)
Respiratory rate (breaths per min), median (IQR)	22 (17–27)
Laboratory findings, median (IQR)	
WBC (1 × 109/l)	7.6 (4.9–12.7)
Neu (1 × 109/l)	4.5 (3.9–10.2)
Lym (1 × 109/l)	0.9 (0.5–1.1)
PLT (1 × 109/l)	173 (132–298)
Hb (g/l)	10.3 (8.6–14.8)
Fibrinogen (g/l)	4.4 (2.1–21.6)
Ferritin (ng/ml)	841 (321–3,348)
CRP (mg/l)	17.8 (4–82)
PCT (ng/ml)	0.6 (0.1–3.3)
LDH (UI/l)	301 (189–506)
ALT (U/l)	55 (34–213)
D-Dimer (ng/ml)	747 (202–1,724)
TNF-a (normal value <14 pg/ml)	2.2 (1–10.6)
MCP-1 (normal range 200–720 pg/ml)	524 (152–1,471)
IL-6 (normal value <12.7 pg/ml)	24.5 (4.5–111)
PaO_2/FiO_2	159 (106–208)

Chicago, IL), *P*-values (two-tailed) below 0.01 were considered statistically significant.

RESULTS

Demographics and Baseline Characteristics

In the time frame of the retrospective observational study, the clinical data of 18 patients (12 males, six females) with confirmed critical COVID-19 were collected. Median age was 62.5 years, range 28–86. All patients were included in the final analysis. The detailed demographic and clinical profile data of all critically ill patients with COVID-19 on baseline are summarized in **Table 2**. Comorbidity was present in six patients (33%) and they all had pre-existing COVID-19 medical conditions and were well-compensated with medical treatment. Distribution by sex reproduced incidence in the Italian population COVID-19 positive (female 33% vs. male 67%).

Primary and Secondary Survey in All Patients

The median time from the beginning of COVID-19 related symptoms and the beginning of ruxolitinib therapy was 9 days (range 4–15). All 18 patients started ruxolitinib treatment on rapidly progressive ARDS, showing a median PaO_2/FiO_2 ratio of 159 (range 106–208) on NIV and being eligible for mechanical ventilation in accordance with the guidelines of our ICU. All patients continued their planned treatments according to the best local practices or guidelines for COVID-19 along with ruxolitinib.

Analysis of our data showed no evolution from NIV to mechanical ventilation in 16/18 patients and no response in two patients. Sixteen out of 18 patients showed a significant improvement in respiratory response within the first 48 h. After 7 days of ruxolitinib treatment, 11/18 patients showed fully recovered respiratory function (pO_2 > 98% in spontaneous breathing), 4/18 patients had minimal oxygen requirement (2–4 L/m) 1/18 patient showed stable disease and 2/18 patient showed progressive disease. At day 14 of ruxolitinib treatment, 16/18 patients showed complete respiratory function. The complete ORR was 89%. In 4/16 responsive patients, the first 2 days of ruxolitinib treatment at full planned dose (20 mg BID) were followed by a faster drug de-escalation (3 days at 10 mg BID, 2 days at 5 mg BID) for a total treatment length of 7 days. As a secondary survey, a rapid restoration of PaO_2/FiO_2 ratio was observed in all responsive patients (16/18) during the first 48 h of ruxolitinib treatment. **Figure 1** and **Table 3** show, in detail,

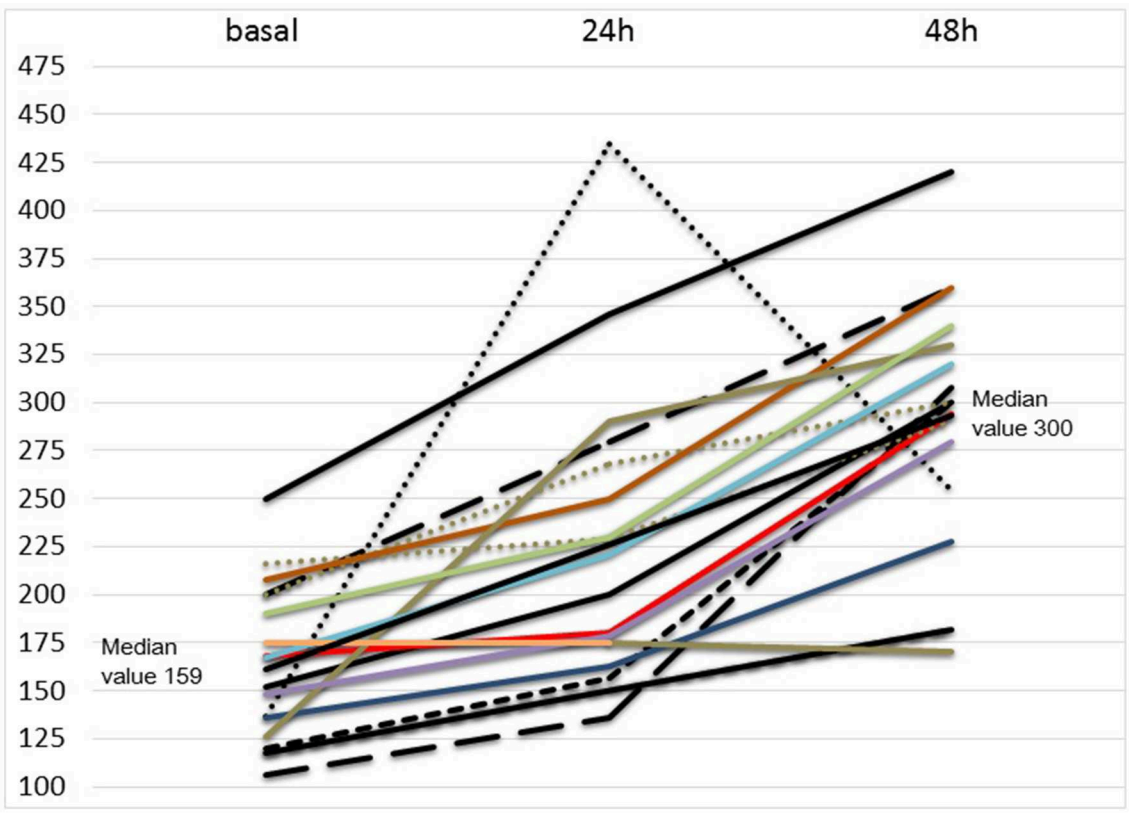


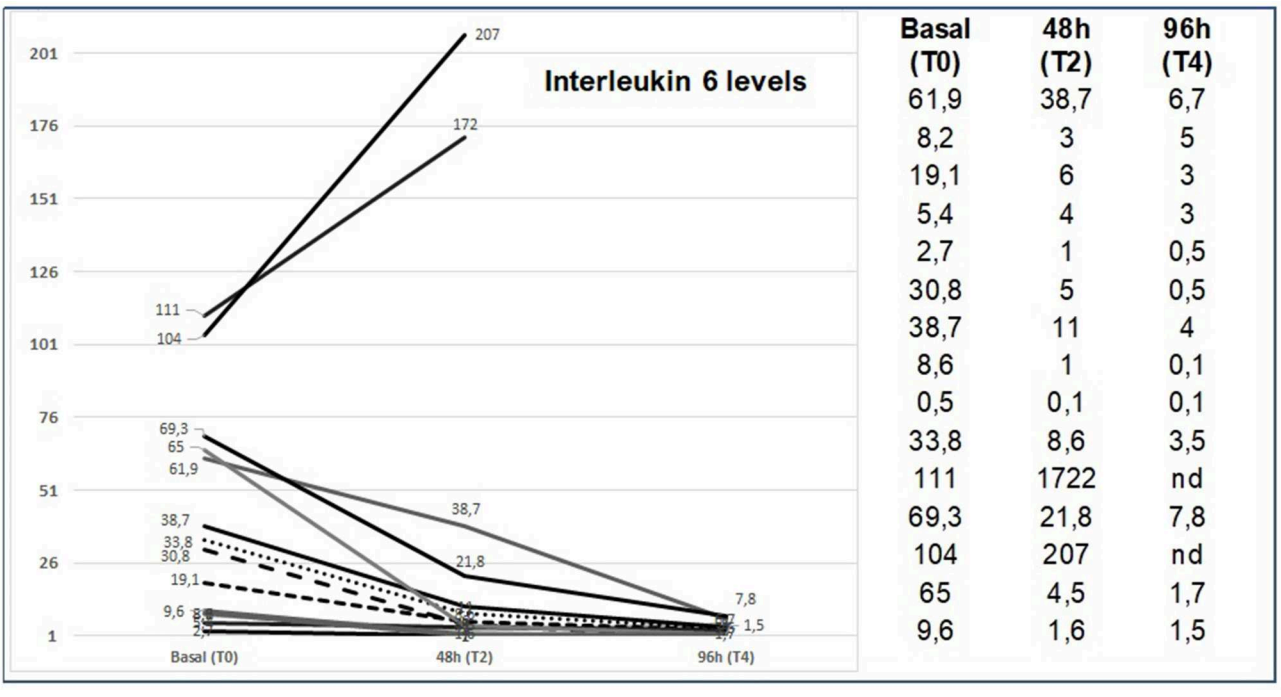
FIGURE 1 | pO_2/FiO_2 ratio in first 48 h of ruxolitinib treatment.

the respiratory data evolution from starting ruxolitinib treatment in all 18 patients. Regarding laboratory findings, neither the reduction of LDH ($p = 0.49$) nor ferritin ($p = 0.7$) correlated with respiratory response. Also, restoration of lymphocyte count ($p = 0.49$) was not related with respiratory response. D-Dimer levels (median 747, range 202–1,724) was at the upper limit in all patients. Normal (pg/ml $<$ 12.7) or high (pg/ml $>$ 12.7) IL6 levels at T0 significantly correlated to time from first COVID-19 symptoms (fever, cough), at fewer or more than 10 days ($p < 0.001$).

Responsive patients (16/18) showed a rapid reduction in IL6 levels (**Figure 2**). On the contrary, the non-responsive patients (2/18) showed a significant IL-6 increase (pts 11: T0 = 111 vs. T2 = 1722, pts 12: T0 = 104 vs. T2 = 286). CRP levels (median 17.8, range 4–82) was at the upper limit in all patients. We saw a statically significant correlation between rapid respiratory response and CRP reduction in the first 48 h, with $p < 0.001$. All patients had good compliance to ruxolitinib, and none discontinued the drug or needed a reduction of the planned dose. No drug related AEs were observed, neither during treatment, nor during follow up after treatment ended. Median follow up after ruxolitinib discontinuation was 21 days (range 7–32). Analysis of the data showed no relevant reductions in leucocytes count, erythrocytes, or platelets. Chest imaging was performed with thoracic ultrasound (TUS) (13/18 pts), chest X Ray (CXR), and computed axial tomography (CT scan) (5/18). Ultrasonographic B lines (19) reduction was observed with a median delay of 2.5 (range 2–5) days compared to the clinical improvement. **Figures 3**, **4** depict CT and CXR imaging from three representative patients. In the same period of time of our observational study, 33 COVID-19 patients with severe respiratory distress were admitted in our ICUs and were not treated with ruxolitinib. The outcome of these patients showed a 19/33 evolution from NIV to mechanical ventilation (57%) and 9/33 patients died (27%).

DISCUSSION

Critical type COVID-19 patients showed poor prognosis. Compared to SARS and MERS, COVID-19 demonstrates several exceptionalities, including prolonged course, potential asymptomatic hypoxia, severe lung injury, and unexpected progression induced death (3). These clinical heterogeneous features suggest pursuing exploratory treatment attempts. JAK-STAT inhibitors are one such attempts. JAK-STAT inhibitors may indeed offer an interesting model of cytokines storm reduction also in the acute respiratory distress syndrome observed in COVID-19 patients. In our case series of 18 critically ill patients with COVID-19 and progressive ARDS, administration of ruxolitinib sensibly ameliorate the course of disease allowing the avoidance of mechanical ventilation in 89% of treated patients. Notably, all patients are alive. On the contrary, evolution from NIV to mechanical ventilation in 33 COVID-19 patients with ARDS treated with the usual ICU guidelines without ruxolitinib was 57% (19 pts) and 27% (9 pts) of them died. Even though we cannot consider these patients as a case control series for our observational study, their outcome data are in accordance with what was recently reported by Grasselli et al.



Basal (T0)	48h (T2)	96h (T4)
61,9	38,7	6,7
8,2	3	5
19,1	6	3
5,4	4	3
2,7	1	0,5
30,8	5	0,5
38,7	11	4
8,6	1	0,1
0,5	0,1	0,1
33,8	8,6	3,5
111	1722	nd
69,3	21,8	7,8
104	207	nd
65	4,5	1,7
9,6	1,6	1,5

FIGURE 2 | IL6 levels at baseline, after 24, 48, and 96 h from starting ruxolitinib treatment.

TABLE 3 | pO_2/FiO_2 ratio and respiratory support type.

	pO_2/FiO_2 ratio			Respiratory support				
Pts	**Basal**	**24 h**	**48 h**	**Basal**	**24 h**	**48 h**	**7 day**	**14 day**
1	118	151	182	cPAP	VM 60%	VM 50%	NC 6L	ra
2	106	136	308	VM 50%	VM 40%	VM 30%	ra	ra
3	152	200	300	cPAP	cPAP	VM 50%	ra	ra
4	120	156	300	cPAP	cPAP	cPAP	VM 30%	ra
5	137	435	255	cPAP	cPAP	MV	cPAP	VM 60%
6	200	280	360	cPAP	VM 50%	VM 30%	ra	ra
7	136	163	228	VM 50%	VM 40%	VM 30%	NC 3L	ra
8	168	180	294	cPAP	VM 50%	VM 30%	NC 3L	ra
9	118	151	182	cPAP 60%	cPAP	VM 50%	VM 30%	ra
10	200	268	300	cPAP	VM 50%	VM 50%	NC 4L	ra
11	216	229	290	cPAP	VM 40%	VM 40%	VM 30%	ra
12	208	250	360	cPAP	VM 50%	VM 50%	ra	ra
13	175	346	420	VM 50%	VM 40%	VM 30%	NC 3L	ra
14	126	290	330	VM 50%	VM 40%	VM 30%	NC 2L	ra
15	190	230	340	cPAP	VM 40%	VM 40%	ra	ra
16	148	178	280	VM 50%	VM 30%	VM 20%	ra	ra
17	167	221	320	cPAP	VM 40%	VM 30%	NC 4 L	ra
18	175	175	170	cPAP	cPAP	MV	cPAP	VM 50%

cPAP, Continuous positive airway pressure; VM, Ventimask; NC, nasal cannula; ra, on room air; MV, mechanic ventilation.

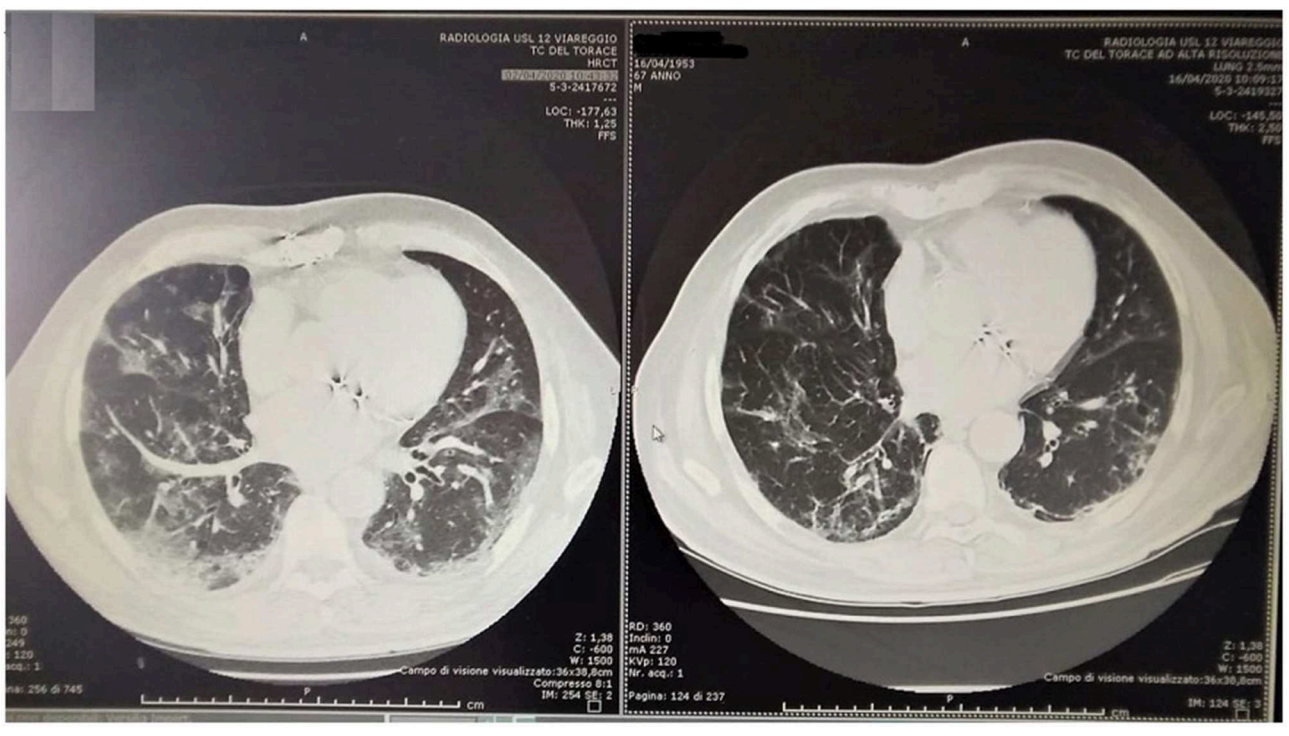


FIGURE 3 | CT chest in 67 years old patient at T0 **(Left)** and at day 14 of treatment **(Right)**.

(7) in a retrospective case series of 1,591 consecutive patients with laboratory-confirmed COVID-19, referred to the COVID-19 ICUs network in Northern Italy. This report confirms that the mortality in Italian ICUs in a COVID-19 setting, similar to that we assessed (median age 63 years, male/female ratio 3:1, comorbidity numbers and baseline PaO_2/FiO_2 with median =

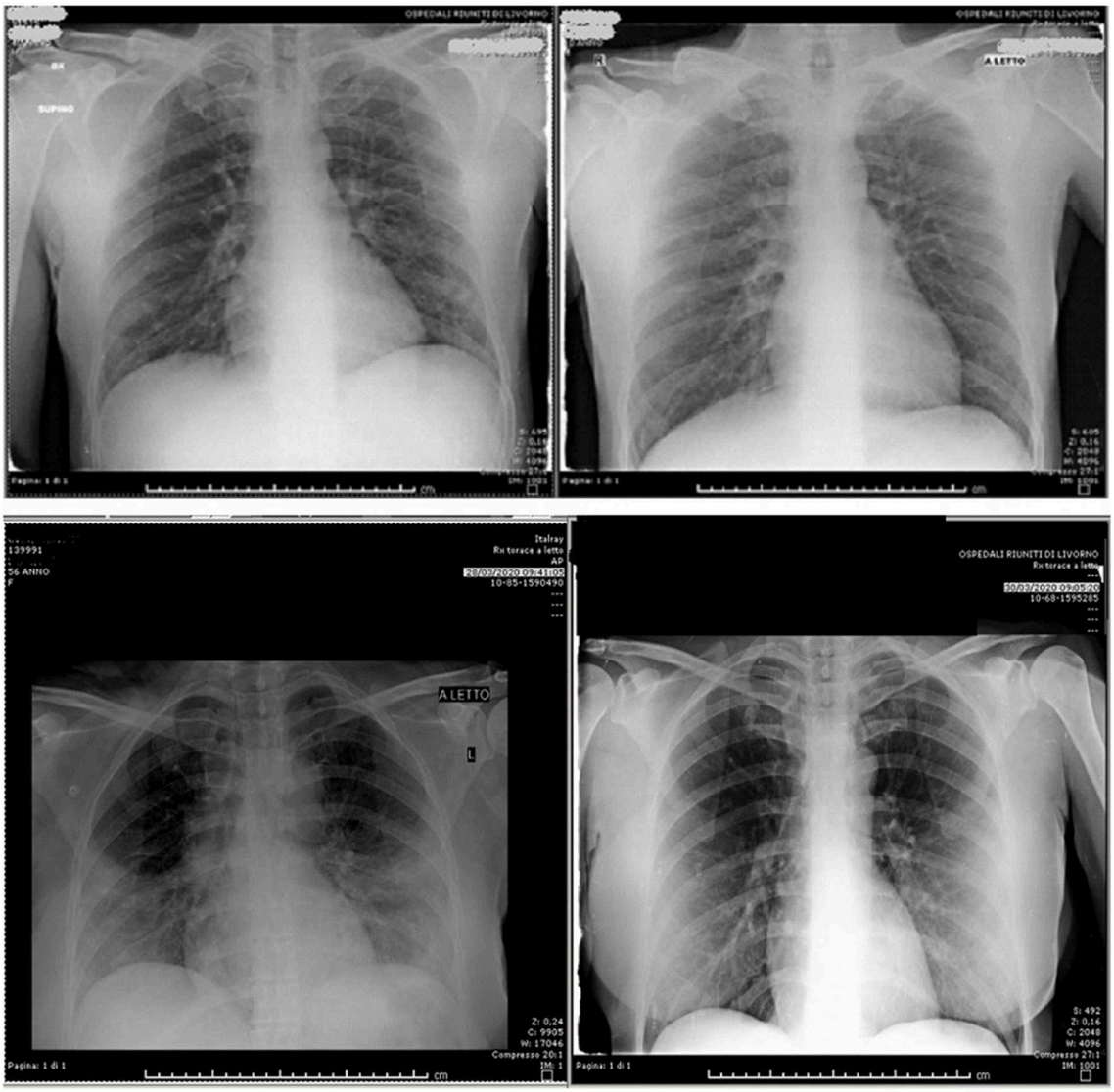


FIGURE 4 | CXR at T0 and after 48 h of ruxolitinib treatment in two patients.

156), was 29% in the age group 60–70 (597 pts) and 40% in the age group 71–80 (340 pts). CRP and IL6 rapid reduction seem to be directly related to clinical improvement and are conceivable to be anticipatory parameters of response. Very recently, La Rosee et al. reported efficacy of ruxolitinib in 14 severe COVID-19 patients prospectively stratified for targeted inhibition of cytokine, using a newly developed COVID-19 Inflammation Score (CIS) (20). The starting doses of ruxolitinib employed in this study were lower (7.5 mg BID) and then increased over time and clinical efficacy, documented in the majority of patients, peaked after 7 days of treatment. This paper confirms the positive effect of ruxolitinib in severe COVID-19 patients with the unique difference that in our series we used a short-term high dose starting schedule, documenting an apparently faster and clinically more relevant response (16/18 patients with significant improvement after 48 h of treatment, 11/18 with spontaneous breathing -complete response- after 7 days of treatment). The rationale to employ higher doses and most likely to achieve a rapid reduction in hyperinflammation was based on the evidence that ruxolitinib demonstrated dose and time depending inhibition of cytokine induced pSTAT3, with maximal inhibition occurring 1 to 2 h from oral intake and with maximal mean inhibition of 40% at 5 mg vs. 90% at 20 mg (21).

Even if recurrently used during this pandemic, the COVID-19 "cytokine storm" is an attractive image behind which there is a really imprecise concept. No one is sure of what the term really means in pathophysiological terms. However, in both La Rosee and our retrospective experiences, the clinical improvement after ruxolitinib appeared related to a quenching of an acute and

rapidly evolving hyperinflammation status. We also believe that one of the most challenging issue in COVID-19 life-threatening disease, is to identify the best timing to initiate ruxolitinib or any other anti-cytokine approach. In treated patients, the interval between the appearance of COVID-19 symptoms and the onset of ruxolitinib treatment was about 10 days, when viral damage subsides and hyperinflammation damage begins. Additionally, we found that the best results in our patients were obtained in those in whom respiratory symptoms were worsening but with still reversible lung damage. Clinical and/or laboratory markers, such as newly reported CIS (20) might be beneficial in defining the right time to initiate the drug and will be a matter of future studies. We are aware that our evaluation of ruxolitinib effect was mainly based on clinical outcome, rather than direct cellular and molecular assessment, including cytokine production by inflammatory cells and viral load. Regarding the latter, we used ruxolitinib in an off label setting with the assumption that JAK inhibitors may play a role in controlling ARDS hyperinflammation with no expected direct effect on viral load. In addition, it could have been very difficult to correlate viral load reduction with ruxolitinib treatment considering that the majority of patients started this drug after a median of 9 days from the onset of COVID-19 related symptoms and most likely at the lower end of the viral load curve (22). Moreover, given the similarities between COVID-19 and SARS-CoV, we may speculate that while virus-induced direct pathogenic effects have an essential role in disease severity, viral load is not correlated with the worsening of symptoms (23).

In conclusion, our study provides clinical evidence for the use of JAK inhibitors in the treatment of SARS-CoV-2 infection, including patient selection and administration timing and dosage. Despite the limited number of patients collected, the results obtained are encouraging and indicate ruxolitinib as a potential therapeutic option for patients with severe COVID-19 related respiratory insufficiency. Several trials exploring the efficacy of ruxolitinib to counteract ARDS in COVID-19 patients just started worldwide (https://clinicaltrials.gov/ct2/results?cond=COVID&term=ruxolitinib&cntry=&state=&city=&dist=) and the cumulative data coming from these studies will be crucial to confirm our observations.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The study was approved to Italian COVID-19 Ethical Committee (National Institute for Infectious Diseases Lazzaro Spallanzani) (trial register no. 81 April 2020). The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

ECa conceived the study and co-wrote the paper. MB co-wrote the paper and supervised data analysis. GI contributed to study design. BF, AP, SSa, PR, AC, FF, FS, DC, IB, DN, RR, SSc, and SV managed patients and collected clinical and laboratory data. ECo and AG analyzed data. All authors approved the manuscript.

ACKNOWLEDGMENTS

Dr. ssa Barbara Meini, Task Force Sperimentazioni Cliniche, Azienda USL Toscana NordOvest.

REFERENCES

1. Zhou P, Yang XL, Wang XG, Hu B, Zhang L, Zhang W, et al. A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature*. (2020) 579:270–73. doi: 10.1038/s41586-020-2012-7
2. Zhou F, Yu T, Du R, Fan G, Liu Y, Liu Z, et al. Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: a retrospective cohort study. *Lancet*. (2020) 395:1054–62. doi: 10.1016/S0140-6736(20)30566-3
3. Xu Z, Shi L, Wang Y, Zhang J, Huang L, Zhang C, et al. Pathological findings of COVID-19 associated with acute respiratory distress syndrome. *Lancet Respir Med*. (2020) 8:420–2. doi: 10.1016/S2213-2600(20)30076-X
4. Ruan Q, Yang K, Wang W, Jiang L, Song, J. Clinical predictors of mortality due to COVID-19 based on an analysis of data of 150 patients from Wuhan, China. *Intensive Care Med*. (2020) 6:1–4. doi: 10.1007/s00134-020-05991-x
5. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet*. (2020) 395:497–506. doi: 10.1016/S0140-6736(20)30183-5
6. Alhazzani W, Moller MH, Arabi YM, Loeb M, Gong MN, Fan E, et al. Surviving Sepsis Campaign: guidelines on the management of critically ill adults with Coronavirus Disease 2019 (COVID-19). *Crit Care Med*. (2020) 48:e440–69. doi: 10.1097/CCM.0000000000004363
7. Grasselli G, Zangrillo A, Zanella A, Antonelli M, Cabrini L, Castelli A, et al. Baseline characteristics and outcomes of 1591 patients infected with SARS-CoV-2 admitted to ICUs of the Lombardy Region, Italy. *JAMA*. (2020) 323:1574–81. doi: 10.1001/jama.2020.5394
8. Channappanavar R, Perlman S. Pathogenic human coronavirus infections: causes and consequences of cytokine storm and immunopathology. *Semin Immunopathol*. (2017) 39:529–39. doi: 10.1007/s00281-017-0629-x
9. Liu J, Zheng X, Tong Q, Li W, Wang B, Sutter K, et al. Overlapping and discrete aspects of the pathology and pathogenesis of the emerging human pathogenic coronaviruses SARS-CoV, MERS-CoV, and 2019-nCoV. *J Med Virol*. (2020) 92:91–4. doi: 10.1002/jmv.25709
10. Zhang H, Zhou P, Wei Y, Yue H, Wang Y, Hu M, et al. Histopathologic changes and SARS-CoV-2 immunostaining in the lung of a patient with COVID-19. *Ann Intern Med*. (2020) 172:629–32. doi: 10.7326/M20-0533
11. Riviere S, Galicier L, Coppo P, Marzac C, Aumont C, Lambotte O, et al. Reactive hemophagocytic syndrome in adults: a retrospective analysis of 162 patients. *Am J Med*. (2014) 127:1118–25. doi: 10.1016/j.amjmed.2014.04.034
12. Mehta P, McAuley DF, Brown M, Sanchez E, Tattersall RS, Manson JJ. COVID-19: consider cytokine storm syndromes and immunosuppression. *Lancet*. (2020) 395:1033–34. doi: 10.1016/S0140-6736(20)30628-0
13. Haberman R, Axelrad J, Chen A, Castillo R, Yan D, Izmirly P, et al. Covid-19 in immune-mediated inflammatory diseases — case series from New York. *N Engl J Med*. (2020) 383:85–8. doi: 10.1056/NEJMc2009567

14. Stebbing J, Phelan A, Griffin I, Tucker C, Oechsle O, Smith D, et al. COVID-19: combining antiviral and anti-inflammatory treatments. *Lancet Infect Dis.* (2020) 20: 400–2. doi: 10.1016/S1473-3099(20)30132-8
15. Spoerl S, Mathew NR, Bscheider M, Schmitt-Graeff A, Chen S, Mueller T, et al. Activity of therapeutic JAK 1/2 blockade in graft-versus-host disease. *Blood.* (2014) 123:3832–42. doi: 10.1182/blood-2013-12-543736
16. Przepiorka D, Luo L, Subramaniam S, Qiu J, Gudi R, Cunningham LC, et al. FDA Approval summary: ruxolitinib for treatment of steroid-refractory acute graft-versus-host disease. *Oncologist.* (2020) 25:e328–34. doi: 10.1634/theoncologist.2019-0627
17. Ahmed A, Merrill SA, Alsawah F, Bockenstedt P, Campagnaro E, Devata S, et al. Ruxolitinib in adult patients with secondary haemophagocytic lymphohistiocytosis: an open-label, single-centre, pilot trial. *Lancet Haematol.* (2019) 6:e630–7. doi: 10.1016/S2352-3026(19)30156-5
18. Phua J, Weng L, Ling L, Egi M, Lim C-M, Divatia JV, et al. Intensive care management of coronavirus disease 2019 (COVID-19): challenges and recommendations. *Lancet Respir Med.* (2020) 8:506–17. doi: 10.1016/S2213-2600(20)30161-2
19. Peng Q-Y, Wang X-T, Zhang L-N. Findings of lung ultrasonography of novel corona virus pneumonia during the 2019-2020 epidemic. *Intensive Care Med.* (2020) 46:849–50. doi: 10.1007/s00134-020-05996-6
20. La Rosée F, Bremer H C, Gehrke I, Kehr A, Hochhaus A, Birndt S, et al. The Janus kinase 1/2 inhibitor ruxolitinib in COVID-19 with severe systemic hyperinflammation. *Leukemia*. (2020) 34:1805–15 doi: 10.1038/s41375-020-0891-0
21. Quintas-Cardama A, Vaddi K, Liu P, Manshouri T, Li J, Scherle PA, et al. Preclinical characterization of the selective JAK1/2 inhibitor INCB018424: therapeutic implications for the treatment of myeloproliferative neoplasms. *Blood*. (2010) 115:3109–17. doi: 10.1182/blood-2009-04-214957
22. Pan Y, Zhang D, Yang P, Poon LLM, Wang Q. Viral load of SARS-CoV-2 in clinical samples. *Lancet Infect Dis*. (2020) 20:411–2. doi: 10.1016/S1473-3099(20)30113-4
23. Peiris JSM, Chu CM, Cheng VCC, Chan KS, Hung IFN, Poon LLN, et al. Clinical progression and viral load in a community outbreak of coronavirus-associated SARS pneumonia: a prospective study. *Lancet*. (2003) 361:1767–72. doi: 10.1016/S0140-6736(03)13412-5

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Medicine

SYSTEMATIC REVIEW
published: 04 August 2020
doi: 10.3389/fmed.2020.00465

Saliva as a Candidate for COVID-19 Diagnostic Testing: A Meta-Analysis

*László Márk Czumbel[1], Szabolcs Kiss[2,3], Nelli Farkas[2], Iván Mandel[4], Anita Hegyi[4], Ákos Nagy[4], Zsolt Lohinai[5], Zsolt Szakács[2], Péter Hegyi[2], Martin C. Steward[1,6] and Gábor Varga[1]**

[1] Department of Oral Biology, Faculty of Dentistry, Semmelweis University, Budapest, Hungary, [2] Institute for Translational Medicine, Medical School, University of Pécs, Pécs, Hungary, [3] Doctoral School of Clinical Medicine, University of Szeged, Szeged, Hungary, [4] Department of Dentistry, Oral and Maxillofacial Surgery, Medical School, University of Pécs, Pécs, Hungary, [5] Department of Conservative Dentistry, Faculty of Dentistry, Semmelweis University, Budapest, Hungary, [6] School of Medical Sciences, University of Manchester, Manchester, United Kingdom

OPEN ACCESS

Edited by:
Nicola Petrosillo,
Istituto Nazionale per le Malattie Infettive Lazzaro Spallanzani (IRCCS), Italy

Reviewed by:
Paolo Antonio Grossi,
University of Insubria, Italy
Gordon Proctor,
King's College London, United Kingdom

***Correspondence:**
Gábor Varga
varga.gabor@dent.semmelweis-univ.hu

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: 02 June 2020
Accepted: 13 July 2020
Published: 04 August 2020

Citation:
Czumbel LM, Kiss S, Farkas N, Mandel I, Hegyi A, Nagy Á, Lohinai Z, Szakács Z, Hegyi P, Steward MC and Varga G (2020) Saliva as a Candidate for COVID-19 Diagnostic Testing: A Meta-Analysis. Front. Med. 7:465. doi: 10.3389/fmed.2020.00465

Background: COVID-19 is a serious and potentially deadly disease. Early diagnosis of infected individuals will play an important role in stopping its further escalation. The present gold standard for sampling is the nasopharyngeal swab method. However, several recent papers suggested that saliva-based testing is a promising alternative that could simplify and accelerate COVID-19 diagnosis.

Objectives: Our aim was to conduct a meta-analysis on the reliability and consistency of SARS-CoV-2 viral RNA detection in saliva specimens.

Methods: We have reported our meta-analysis according to the Cochrane Handbook. We searched the Cochrane Library, Embase, Pubmed, Scopus, Web of Science and clinical trial registries for eligible studies published between 1 January and 25 April 2020. The number of positive tests and the total number of tests conducted were collected as raw data. The proportion of positive tests in the pooled data were calculated by score confidence-interval estimation with the Freeman–Tukey transformation. Heterogeneity was assessed using the I^2 measure and the χ^2-test.

Results: The systematic search revealed 96 records after removal of duplicates. Twenty-six records were included for qualitative analysis and 5 records for quantitative synthesis. We found 91% (CI 80–99%) sensitivity for saliva tests and 98% (CI 89–100%) sensitivity for nasopharyngeal swab (NPS) tests in previously confirmed COVID-19 patients, with moderate heterogeneity among the studies. Additionally, we identified 18 registered, ongoing clinical trials of saliva-based tests for detection of the virus.

Conclusion: Saliva tests offer a promising alternative to NPS for COVID-19 diagnosis. However, further diagnostic accuracy studies are needed to improve their specificity and sensitivity.

Keywords: coronavirus, SARS-CoV-2, COVID-19, diagnostic tests, saliva, systematic review, meta-analysis

INTRODUCTION

COVID-19, caused by the SARS-CoV-2 virus, is a serious and potentially deadly disease. Globally, as of 5 May 2020, there have been 3,489,053 confirmed cases of COVID-19 reported to WHO, including 241,559 deaths (1). Early diagnosis and isolation of infected individuals will play an vital role in stopping the further escalation of the pandemic.

At present, nasopharyngeal swabbing, followed by reverse transcription of the extracted RNA and quantitative PCR (RT-qPCR), is the gold standard for detection of SARS-CoV-2 infection (2). Specimen collection currently requires trained medical personnel (3), thus exposing staff to a high risk of infection (4). These tests are not always successful at the first attempt, and shortages of swabs and protective equipment are frequently reported (2). Additionally, mass testing requires an increased number of trained personnel at specimen acquisition sites. Consequently, the nasopharyngeal swab (NPS) collection method is causing an economic and logistic burden on healthcare systems. Additionally, nasopharyngeal swabbing causes discomfort to the patients (5) and there are several contraindications, such as coagulopathy or anticoagulant therapy, and significant nasal septum deviation (6). Clearly, there is a need for a simpler and less invasive method that also reduces the risk to healthcare personnel.

One candidate for non-invasive specimen collection is saliva. The saliva secreted by salivary glands contains water, electrolytes, mucus, and digestive and protective proteins (7–9). But whole saliva collected from the mouth is a mixture of glandular secretions, gingival crevicular fluid, serum, expectorated airway surface liquid and mucus, epithelial and immune cells from the oral mucosa and upper airways, and oral microbes and viruses (10). Despite its heterogeneous origins, this mixed fluid is used widely and successfully as a diagnostic tool to identify various oral and systemic conditions (8, 11). These already include viral infections such as dengue, West Nile, chikungunya, Ebola, Zika and Yellow Fever, and also the recently emerged coronaviruses responsible for severe acute respiratory syndrome (SARS) and Middle East respiratory syndrome (MERS) (12).

Since early January 2020, several papers have been published on the possible use of saliva as a specimen for detecting SARS-CoV-2 in the diagnosis of COVID-19. Until now there has been no systematic review or meta-analysis of this topic. Our aim, therefore, was to conduct a meta-analysis, thus overcoming the limitations of the small sample sizes in individual studies, in order to estimate the diagnostic sensitivity of saliva-based detection of the virus. We also aimed to summarize the study protocols that have been registered in clinical trial registries to investigate saliva-based COVID-19 diagnosis in the future.

MATERIALS AND METHODS

Protocol and Registration

The reporting of our meta-analysis follows the guidelines of the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) (13). The PRISMA checklist for our work is available in the supporting information (**Table S1**). We registered our meta-analysis protocol in the OSF (Open Science Framework, Center for Open Science) registries on 23 April 2020 (https://osf.io/3ajy7).

Deviation From the Registered Protocol

Studies eligible according to our inclusion criteria did not present sufficient raw data to complete 2 × 2 contingency tables. True positive, true negative, false positive and false negative values were not generally available, thus sensitivity and specificity could not be separately calculated. Instead, positive event rates were pooled for statistical analysis. Details of the analysis are described in section Summary Measures and Synthesis of Results.

Eligibility Criteria

We included records if they met the following eligibility criteria: (1) records published in scientific journals or clinical trial registries; (2) patients diagnosed with COVID-19; (3) index test: saliva specimens with PCR diagnostics for detecting SARS-CoV-2; (4) reference standard (comparator test): NPS specimens with PCR diagnostics for detecting SARS-CoV-2; (5) records written in English or available in English translation. Exclusion criteria: (1) publications with no primary results such as reviews, guidelines and recommendations; (2) publications dated before 1 January and after 25 April, 2020; (3) gray and black literature.

Search Strategy

Systematic searches for records published in English after 1 January 2020 were performed in five major literature databases (Cochrane Library, Embase, PubMed, Scopus, Web of Science) and also in five clinical trial registers (ClinicalTrial.gov, EU Clinical Trials Register, NIPH Clinical Trial Search, ISRCTN Registry, ANZCTR Registry). The last update of our systematic search was performed on 25 April 2020. Cited and citing papers of the relevant studies were screened for further eligible studies.

The following key words were applied to each database to identify eligible records: (COVID 19 OR COVID19 OR Wuhan virus OR Wuhan coronavirus OR coronavirus OR 2019 nCoV OR 2019nCoV OR 2019-nCoV OR SARS CoV-2 OR SARS-CoV-2 OR NCP OR novel coronavirus pneumonia OR 2019 novel coronavirus OR new coronavirus) AND (saliva).

Study Selection

We used EndNote X9.3.3 reference manger to organize records. After removal of duplicates, two authors (A.H. and I.M.) independently screened the records for eligibility based on the titles and abstracts. Papers included at this stage were further appraised by reading the full text. Any disagreement between reviewers was resolved by consulting a third reviewer (L.M.C.).

Data Collection

Using a preconstructed, standardized data extraction form, two authors (A.H. and I.M.) independently collected data from the included records. From primary studies the following information was extracted (**Table 1**): first author's name, year of publication, place of study, study type, population size, age, gender, method of diagnosis, type of PCR kit, and the following outcome parameters: numbers of total, positive and negative saliva tests and numbers of total, positive and negative NPS

TABLE 1 | Summary of study characteristics of included records.

References	Country	Study type	Population		Diagnoses of COVID-19	PCR kit	Reference standard	Index test	Outcome parameters
			n (m/f)	Age					
(14)	Italy	Consecutive case series	25 (17/8)	61 (mean) (39–85)	Viral RNA detection with PCR from NPS	Luna Universal qPCR Master Mix	NPS	Saliva	Number of positive and negative index tests
(15)	South Korea	Consecutive case series	4 (2/2)	61.5 (35–82)	Viral RNA detection with PCR from NPS And clinical signs of pneumonia	N/A	NPS	Saliva	Number of positive and negative index tests
(16)	China	Consecutive case series	32 (16/16)	41 (34–54)	Viral RNA detection with PCR from NPS	N/A	NPS	Saliva	Number of positive and negative index tests
(17)	Hong Kong, China	Consecutive case series	23 (13/10)	62 (37–75)	Viral RNA detection with PCR from NPS	QuantiNova Probe RT-PCR Kit	NPS	Saliva	Number of positive and negative index tests
(18)	Australia	Consecutive case series	39 (not published)	Not published	Viral RNA detection with PCR from NPS	Coronavirus Typing (835 well) assay	NPS	Saliva	Number of positive and negative index tests
Not included in quantitative synthesis:									
(19)	China	Case report	1 (0/1)	39	Viral RNA detection with PCR from NPS And clinical signs of pneumonia	N/A	NPS	Saliva	Number of positive and negative reference tests and index tests
(20)	South Korea	Case report	1 (0/1)	Neonate (27 day-old)	Viral RNA detection with PCR from NPS	PowerChek TM 2019-nCoV Real-time PCR Kit	NPS	Saliva	Number of positive and negative reference tests and index tests
(21)	USA	Consecutive case series	29 (16/13)	59 (mean) (23–91)	Viral RNA detection with PCR from NPS	The US CDC real-time RT-PCR primer/probe sets	NPS	Saliva	Number of positive and negative reference tests and index tests

NPS, Nasopharyngeal swab; N/A, Not available.

tests. From registered study protocols the following information was extracted (**Table S2**): clinical trial ID, recruiting status, study type, number of centers, study design, location, population, intervention, comparison, primary outcomes, and secondary outcomes. In cases of disagreement during extractions a third author (L.M.C.) was consulted.

Risk of Bias and Applicability Assessment

We evaluated the potential for bias, the quality of reporting and the applicability of the studies using the QUADAS-2 tool (Quality Assessment of Diagnostic Accuracy Studies 2) (22), which is a tool widely used to assess studies of diagnostic accuracy. Our appraisal consisted of evaluating the risk of bias and applicability in four domains: (1) patient selection, (2) conduct and interpretation of the index test, (3) reference standard, and (4) flow and timing. We applied the following review question to judge the applicability of the studies to our investigation: *Are saliva specimens reliable for detecting SARS-CoV-2 in COVID-19 patients confirmed by nasopharyngeal swab testing?*

We used the preconstructed form available on the QUADAS-2 web page of the University of Bristol (23).

Summary Measures and Synthesis of Results

In the synthesis of quantitative data we included patient-based data from consecutive case series. Case reports from single participants were excluded.

The sensitivities of the saliva and NPS tests were assessed in patients who had previously been confirmed to be infected, having had both a positive NPS test and well-defined clinical symptoms on admission to the hospital. Extracted data were limited to test results from subsequent occasions when both saliva and NPS samples were collected concurrently. Therefore, the sensitivity of the NPS test is based on the matching NPS tests when saliva tests were also performed.

The sensitivity of the saliva test in the patient-based pooled data was calculated using the methods recommended by the working group of the Cochrane Collaboration. Because some of the sensitivity values are close to or equal to 1, the score confidence interval estimation (24) was applied with the Freeman–Tukey double arcsine transformation (25). Because of the variability of the population sizes and methodologies in the different studies, the DerSimonian and Laird method (26)

was used, with 95% confidence intervals (CI), for a random-effects meta-analysis.

Heterogeneity was assessed using the I^2 measure and the χ^2-test, where $p < 0.1$ is taken to indicate significant heterogeneity. I^2 values of 25, 50, and 75% were identified as low, moderate and high estimates, respectively (27). Statistical analyses were carried out using STATA software version 15.0 (STATACorp, Texas, USA).

RESULTS

Study Selection

We included 20 articles for full-text evaluation of completed studies. Of these, eight were included in the qualitative synthesis, from which five were also included in the quantitative synthesis. **Figure 1** illustrates the study selection process.

Our search in the clinical trial register yielded 19 protocols, of which one was excluded due to its relating to a different topic.

Study Characteristics

Characteristics of the Studies Included

All five records included in the quantitative synthesis were consecutive case series, involving 123 patients from five distinct global locations (**Table 1**) (14–18). All of these publications included patients with confirmed diagnoses of COVID-19. No other restrictions on inclusion were stated in any of the studies.

In the qualitative synthesis we also included another consecutive case series (**Table 1**). But in their work Wyllie et al. presented 38 matching NPS and saliva samples from 29 patients without identifying the double or multiple samplings from individual patients. Therefore, their sample-wise results cannot be combined for quantitative analysis with the others which reported patient-wise data (21).

Results of Individual Studies and Synthesis of Results

Diagnostic Potential of Saliva Specimens

In the individual studies included in the quantitative synthesis, the sensitivity of the saliva test among COVID-19 infected patients ranged from 78% (16) to 100% (14).

Pooled event rates (positive and negative test results) from saliva specimens show that the sensitivity of the saliva test was 91% (CI 80–99%) among COVID-19 patients diagnosed in the recruitment period (**Figure 2A**). By definition, the nature of the initial diagnosis implies or rather assumes a 100% sensitivity for the nasal swab test in those patients at that time point. However, pooled event rates from NPS specimens taken concurrently with the saliva specimen collections, generally some time after the initial diagnosis, indicate that the sensitivity of the NPS test, based on these time-matched samples, was 98% (CI 89–100%) (**Figure 2B**). Since the two confidence intervals overlap, it appears that the proportions of positive test results from the saliva and NPS samples are not very different. However, a firm conclusion will require formal diagnostic accuracy tests based upon larger clinical studies.

We assessed our pooled results for inconsistency using the I^2-test (28). In the case of the saliva tests we found a moderate level of heterogeneity ($I^2 = 60.98\%$, $p = 0.04$) indicating the contribution of confounding factors. On the other hand, we found a low level of heterogeneity among the NPS test results ($I^2 = 46.56\%$, $p = 0.13$).

Interestingly some of the data suggest that NPS tests may occasionally be negative when the corresponding saliva test gives a positive result. Azzi et al. reported that two patients showed positive saliva tests while their NPS tests were negative (14), and a case report showed that in seven sample pairs from one individual, the NPS tests were all negative while the saliva tests were positive on each occasion (19). In a sample-based study of 38 patients, Wyllie et al. (21) detected SARS-CoV-2 in saliva but not NPS specimens from eight patients (21%), while the virus was detected in NPS but not saliva in only 3 matched samples (8%). And overall, they found significantly higher SARS-CoV-2 titers in the saliva than in the NPS specimens.

In a more detailed study, Bae et al. examined the difference in viral loads between the two sampling methods: the values were 0.06 to 3.39 $\log_{10}$ units higher in the NPS specimens than in the saliva specimens (15). One case series (18) and another case report on a 27-day-old neonate (20) also found that there were higher viral loads in the NPS specimens.

Only two studies assessed the specificity of the saliva tests (18, 21). In one, a subset of saliva specimens from 50 patients with PCR-negative nasal swabs was tested. SARS-CoV-2 was detected in 2% (CI 0.1–11.5%) of these saliva samples (18). The other study tested 98 asymptomatic healthcare workers with parallel NPS and saliva tests. NPS tests turned out to be negative for all participants, while saliva tests were positive for two (21).

Risk of Bias Within Studies

We assessed the risk of bias in the six included case series (14–18, 21) according to the QUADAS-2 tool. Five of the six (14–17, 21) had low risk of selection bias. On the other hand four studies (14–17) had high risk of bias in the index test due to the fact that the saliva tests results were interpreted with prior knowledge of the results of the reference standard. Flow and timing were high or unclear in all studies, since there was no exact information regarding the time passed between specimen collections for the two tests. Applicability had low concerns in index test in four studies (14, 17, 18, 21) and unclear in two studies (15, 16). A summary of the risk-of-bias analysis and applicability concerns is available in **Tables S3**, **S4**. Altogether, our risk-of-bias analyses demonstrated a moderate bias level in both the individual and the overall aspects of the studies.

Ongoing Registered Clinical Trials on Saliva Diagnostics for COVID-19

We also systematically searched five clinical trial registers (EU Register, ISRCTN, ANZCTR, JPRN, ClinicalTrials.gov) for clinical trial protocols that are planned to evaluate saliva specimens for COVID-19 diagnosis. By using the same keywords as for the studies already completed, we found 18 registered clinical trials on planned or ongoing clinical studies. All of them appeared in the ClinicalTrials.gov registry (**Table S2**). Among these, 13 are non-interventional, focusing primarily

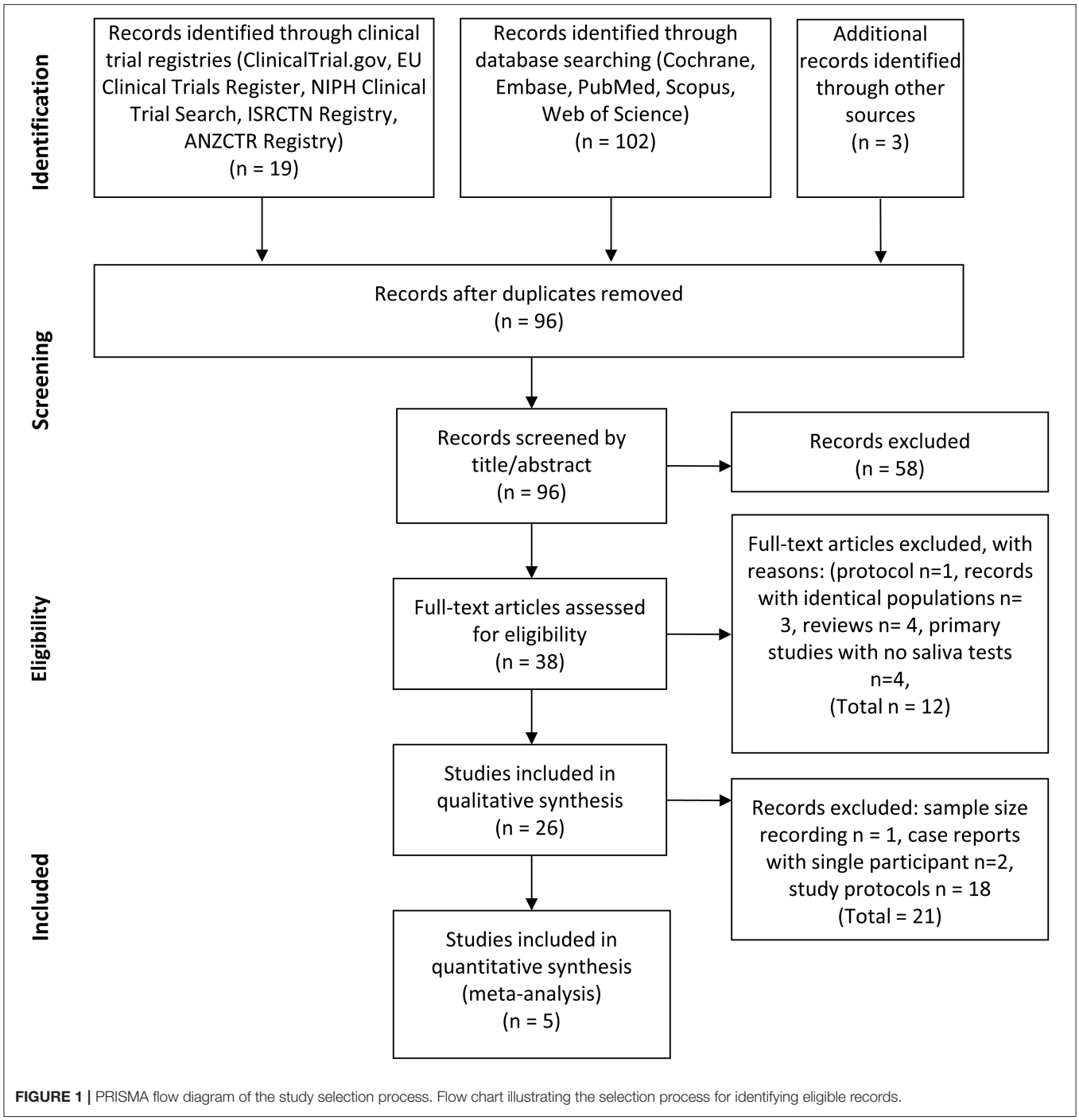


FIGURE 1 | PRISMA flow diagram of the study selection process. Flow chart illustrating the selection process for identifying eligible records.

on the diagnostic and prognostic value of various specimens collected from patients, including NPS, saliva and blood, in detecting and following the progression of COVID-19 disease. The other five, interventional studies are examining the effectiveness of several potentially beneficial compounds, including azithromycin, lopinavir/ritonavir, beta-cyclodextrin, citrox 3 and peginterferon lambda, on the outcomes of SARS-CoV-2 infection. In these studies, besides NPS specimen collections, saliva tests are also planned. Unfortunately, in the trial protocols very little information is available about the optimization and validation of the saliva collection protocols, the transportation and storage of the saliva samples, the viral RNA assay methods to be used for the saliva samples, and the choice of appropriate internal controls, which is important given the scarcity of human DNA in saliva samples.

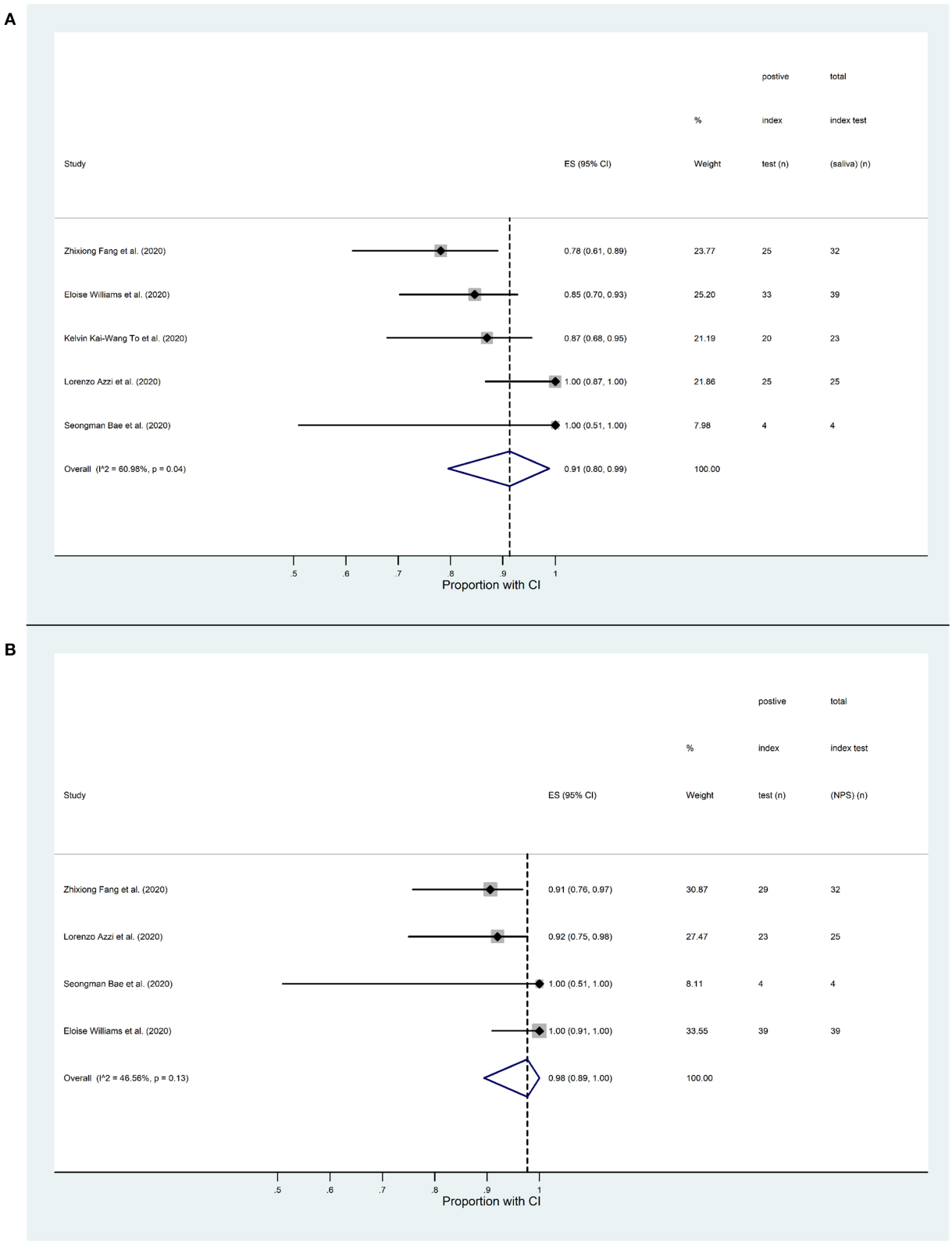


FIGURE 2 | Forest plot analysis of SARS-CoV-2 detection sensitivity based on RT-qPCR analysis of saliva and nasopharyngeal swab (NPS) specimens from COVID-19 patients. **(A)** Proportion of positive saliva tests in the five studies included in the quantitative analysis, ranging from 0.78 to 1. The overall proportion in the pooled data is 0.91 (CI 0.80–0.99). The I^2 value (60.98%, $p = 0.04$) indicates a moderate level of statistical heterogeneity. **(B)** Proportion of positive NPS tests in the four studies included in the quantitative analysis, ranging from 0.91 to 1. The overall proportion in the pooled data is 0.98 (CI 0.89-1). The I^2 value (46.56%, $p = 0.13$) indicates a low level of statistical heterogeneity.

DISCUSSION

In April 2020 the Food and Drug Administration (FDA) granted emergency use authorization (EUA) to Rutgers' RUCDR Infinite Biologics and its collaborators for a new specimen collection approach that utilizes saliva as the primary test biomaterial for the SARS-CoV-2 coronavirus, the first such approval granted by the federal agency (https://www.fda.gov/media/136877/download). This new saliva-based diagnostic collection method, which RUCDR has developed in partnership with Spectrum Solutions and Accurate Diagnostic Labs (ADL), claims to allow an easier and therefore broader screening of the population compared with the current method using nose and throat swabs. Another accelerated EUA for the "Curative-Korva SARS-Cov-2 Assay," which was specifically designed for use with oral fluid samples, was also approved to permit the testing of oral fluids, i.e., saliva (https://www.fda.gov/media/137088/download). Nasopharyngeal swabs, oropharyngeal swabs and nasal swabs can also be used with the Curative-Korva SARS-CoV-2 Assay, but their performance with this assay has not yet been assessed (https://www.fda.gov/media/137088/download). These two saliva-based, FDA-approved assays are now in use to test for COVID-19 infection, in spite of the fact that no independent, scientific analysis has yet established their effectiveness. Our present work is the first integrative meta-analysis study to review the existing multi-study evidence for validity of the saliva-based approach.

The use of saliva as a diagnostic tool for various systemic conditions is nothing new. Considerable research effort has been made in the past to seek biomarkers in saliva, since its collection is non-invasive and easy. As a result, emerging evidence indicates that whole saliva can be used to identify various oral and systemic conditions [for reviews see (8, 11, 29)]. Importantly, the concept of using saliva to detect viral infections is now well-established (12, 30).

Among RNA viruses, salivary diagnostic tests for Zika are well-established (31, 32) and a number of salivary-based detection methods have been reported for Ebola virus detection (12). The presence of considerable quantities of viral RNA in the saliva of 17 SARS-infected patients has also been shown unequivocally (33). But most studies lack any direct comparison of the sensitivity and specificity of NPS- and saliva-based assays. The one important exception is a study which compared saliva and NPS specimens for the detection of respiratory viruses by multiplex RT-PCR (4). This study, which included results from 236 patients with 11 different viral respiratory infections, including coronaviruses, revealed no significant difference in the sensitivity and specificity of saliva- and NPS-based tests (4). Taken together, although saliva-based diagnostics are supported by a considerable amount of evidence, routine applications are still rare because of the lack of well-standardized protocols.

The source of SARS-CoV-2 in saliva is unknown at present but it could come from multiple locations. One obvious source is debris from the nasopharyngeal epithelium which drains into the oral cavity (17). Secondly, SARS-CoV-2 may actually infect the salivary glands and the virus is then secreted into the saliva from the glands. No information is available on this. But it is of note that during the infection of rhesus macaques by the SARS coronavirus, epithelial cells lining salivary gland ducts are an early target of the virus (34). One consequence of this is the production of SARS-specific secretory immunoglobulin A into the saliva (35). Thirdly, SARS-CoV-2 from blood plasma may access the mouth via the crevicular fluid, an exudate derived from periodontal tissues (36). Fourthly, infected oral mucosal endothelial cells, which show overexpression of ACE2 during SARS-CoV-2 infection, may also contribute to the viral load in saliva (37). Finally, salivary cells may endocytose viruses and virus-containing exosomes from the circulation at their basolateral surface and release them into the salivary lumen by exocytosis. Such mechanisms have been revealed for other macromolecular constituents of the blood, such as DNA and RNA (8). Any or all of these five possible sources may contribute to the appearance of SARS-CoV-2 in the saliva of COVID-19 patients. Given also that the main sites of viral infection (nasal, oral, pharyngeal or respiratory tract) may differ between individuals, it is quite possible that in some patients the virus is more readily detected in the saliva and in others it is more readily detected in an NPS specimen. Such differences might also be related to genomic variations between patients (38). Consequently discrepancies between NPS and saliva test results, rather than indicating a deficiency in one or other test, may be an expected outcome, and it may have implications in terms of assessing asymptomatic carriers (39, 40). Either way, our present level of understanding paves the way for more intensive studies of these important issues, extending well-beyond the design of better diagnostics for SARS-CoV-2 infection (6, 38).

In the present meta-analysis we found that the test sensitivities for SARS-CoV-2 were 91% (CI 80–99%) and 98% (CI 89–100%) for saliva and for NPS samples, respectively, based the pooled event rates among COVID-19 patients. Clearly the two confidence intervals overlap, suggesting that the outcomes of the saliva tests and NPS tests are not very different. There appears to be a slight tendency for NPS tests to be more sensitive but this is not statistically significant. On the other hand, one study reported the opposite tendency with the virus detectable in the saliva but not the NPS sample on a significant number of occasions (21). Although NPS-based SARS-CoV-2 virus detection is currently regarded as the gold standard (2, 41, 42), carefully performed future studies need to be carried out to determine the relative diagnostic accuracies and specificities of the saliva and NPS tests.

At present only two studies have considered the specificity of the saliva tests. In one of those tests only one saliva sample was found to be positive among 50 apparently healthy individuals who were PCR-negative for the NPS test (18). In the other work two individuals were detected positive in saliva tests on 98 participants who were negative for NPS test (21). These results may reflect a real difference in the specificities of the NPS and saliva tests, or they may simply be a consequence of occasional false negatives in the NPS tests.

For optimal saliva-based testing at least three conditions have to be improved by standardization and validation (43). (1) A specific saliva collection method should be selected and optimized after systematically comparing the various methods currently used for collecting whole saliva in other clinical

and scientific contexts. (2) The optimal solution for collecting, transporting and storing saliva samples should be found. (3) The RNA assay method, either RT-qPCR, loop-mediated isothermal amplification (LAMP) or another protocol, should also be optimized for saliva, using an appropriate internal control; this cannot be human DNA which is overwhelming in NPS but not in saliva samples (15–18, 21). In order to obtain a reliable and sensitive saliva test, all of these conditions must be standardized.

Not surprisingly the studies included in our analysis used different sampling methods to collect saliva. This may have had a significant effect on the sensitivity of the saliva test. Azzi et al. used a simple drooling technique to collect saliva and they resuspended the collected specimens in 2 ml of PBS (14). In contrast, To et al. collected saliva specimens that also contained fluid from the posterior oropharynx obtained by coughing up and clearing the throat (17). Another study (18) asked patients to pool saliva in their mouth prior to collection, and to spit 1–2 ml into a collection pot. The act of pooling saliva in the mouth may have stimulated additional saliva secretion, which could have diluted the viral load in the specimen. In this case no transport medium was added to the specimens but, after transportation to the laboratories, liquid Amies medium was added. Wyllie et al. used a self-collection technique: patients were asked to spit repeatedly into a sterile urine cup until one third was full (21). This too could have diluted the sample with additional virus-free saliva. The remaining two studies did not describe the collection method at all (15, 16). Additionally, two of the studies specified that specimens were collected in early morning to avoid anomalies introduced by eating, drinking and tooth brushing (17, 21). The rest of the studies did not specify the time of collection or mention any other confounding factors that may have affected the sample. Taken together, the sample collection protocols of the included studies are quite diverse. But it is promising that even without validated, standardized collection protocols, the studies reviewed here yielded very similar results.

Other factors, such as the type of transport medium, the temperature during transportation, and the time passed between specimen collection and RNA extraction, may also affect the outcome of the tests (43). Unfortunately, there is insufficient information in these few studies to draw any conclusions about the possible effects of these confounding factors on the accuracy of saliva testing for COVID-19 diagnosis (15–18, 21). But again, although the five studies used different RNA isolation methods, and different PCR primers and conditions, it is encouraging to note that the virus could in all cases be detected in saliva samples with a consistently high level of sensitivity.

It is likely that a simple drooling technique, with no specific target volume and no extra stimulation of saliva secretion, will provide the greatest sensitivity if the viral RNA in whole saliva derives mainly from sources other than the secretions of the salivary glands. Drooling is a well-established saliva collection method that is generally recommended for analytical purposes (44). Due to its simplicity, it does not require trained personnel, it can be self-administered, and it can be done at home if necessary. Even in the clinic, the drooling method is safer than nasopharyngeal or oropharyngeal swabbing, with no need for infected swabs to be carried through the air from the patient to the container. The fact that nasopharyngeal swab sampling sometimes has to be repeated in overt COVID-19 patients before a positive result is obtained suggests that the reliability of that sampling method is lower than might be expected from saliva sampling. Moreover, this saliva collecting technique also avoids the mixing of fluids from different anatomical regions such as the oropharynx (14).

In the present meta-analysis the overall sensitivity of the saliva (index) test is assessed by comparison with the NPS (reference standard) test using patient-based pooled data. This simple comparison does not allow us to address any of the more complex questions that arise from the widely varying presentation of different COVID-19 patients. For example, are there significant differences in the sensitivities of the two sampling methods according to the primary location of the infected cells? Are there higher viral loads in the saliva, and is there therefore a higher saliva test sensitivity, in COVID-19 patients who only present with a loss of taste sensation or who are asymptomatic? Are saliva tests more or less sensitive than NPS tests in patients whose infection is mainly localized to the respiratory tract? Correlation studies comparing saliva and NPS viral loads in patients categorized by the nature and severity of their symptoms should be very informative. Time series data on the relative viral loads in the saliva and NPS specimens may be useful in predicting the progression of the disease and in guiding treatment. But, as discussed above, these studies will require careful optimization and standardization, particularly of the saliva collection protocol.

The need for reliable, non-invasive and easy-to-perform tests for COVID-19 has focused special attention on saliva in the last few months. Between 1 January and 25 April 2020, 18 clinical trials involving saliva specimens have started according to the ClinicalTrials.gov registry (**Table S2**). Among these, 13 are non-interventional, focusing on the diagnostic value of various specimens including saliva, and five interventional studies also planned to use saliva as a diagnostic tool, but with a primary focus on evaluating potential treatments for SARS-CoV-2 infections. Unfortunately, these registered clinical trials vary considerably in the amount of information presented about the proposed testing methodology. Neither the non-interventional nor the interventional protocols have clear descriptions of the collection, transportation and storage of saliva samples, and the optimization of the viral RNA assay for saliva specimens. Only a few of them emphasize the necessity for determining the sensitivity and specificity of the saliva-based test. But hopefully, during the course of execution, such studies will yield high quality, reliable data that can be used to address some of the important biological and methodological questions that we have discussed here.

LIMITATIONS

A limitation of the present work is the relatively small number of studies and small sample sizes available regarding this topic. Despite the large number of records found in the systematic search of the literature, only 6 studies could be included. Although intensive research is in progress regarding COVID-19, there are still only a handful articles fulfilling our eligibility criteria. The limited amount of reported data makes it difficult to perform comprehensive analyses and to thoroughly investigate

the causes behind certain trends in the results. Another issue that hinders in-depth analysis is the lack of methodological homogeneity, and the inadequate reporting of methods and outcome parameters. A significant limitation is the lack of false-positive data, based on an independent reference, that would be required for 2 × 2 contingency tables to allow estimation of the test specificities. Thus, the more rigorous statistical methodologies specially developed for meta-analysis of diagnostic test accuracy could not be used in this work.

All studies except two (18, 21) investigated the reliability of the saliva test only among confirmed COVID-19 infected participants, with no healthy individuals or asymptomatic COVID-19 patients recruited for comparison. Additionally, there are several other confounding factors that might have affected the detectability of viral RNA in the saliva, such as the timing and method of sample collection, the choice of transport medium, storage and transport temperatures, the time passed between specimen collection and RNA isolation, and the extraction and PCR kits used for isolation, amplification and detection. None of these factors could be properly addressed in our analysis owing to the lack of information in the reported studies.

CONCLUSION

In the present meta-analysis we provide evidence that saliva tests are a promising alternative to nasopharyngeal swab tests for COVID-19 diagnosis. Optimized and validated saliva assays offer the possibility of reliable self-collection of samples for COVID-19 testing in the future. However, there are many open questions to be answered before the precise specificity and sensitivity of the saliva-based tests can be determined and appropriate standardized procedures introduced into clinical practice.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: The data that support the findings of this study are available from the corresponding author, upon reasonable request.

AUTHOR CONTRIBUTIONS

LC, SK, ÁN, ZL, ZS, PH, MS, and GV devised the project, the main conceptual ideas and planned the research. LC, SK, NF, ZS, and GV worked out the methodology. IM, AH, and LC performed the data collection, literature search, study selection, and data extraction. LC, IM, and AH also organized and maintained research data for analysis. NF performed analytic calculations and applied statistical models for synthetizing data. ZS and SK also aided the research by interpretation of raw and synthetized data. NF visualized synthetized data into forest plots. LC and GV worked on summarizing results into figures and tables. SK and GV were responsible for managing and coordinating the research activity. PH and GV took leadership responsibility for the research activity, provided resources, and acquired financial support for the research project. ZS, PH, MS, and GV validated reproducibility of the results. LC, NF, IM, AH, and GV wrote the manuscript with input from all authors. SK, ÁN, ZL, ZS, PH, MS, and GV extensively reviewed the work and further edited the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by the Hungarian Human Resources Development Operational Program (EFOP-3.6.2-16-2017-00006). Additional support was received from an Economic Development and Innovation Operative Program Grant (GINOP 2.3.2-15- 2016-00048) and an Institutional Developments for Enhancing Intelligent Specialization Grant (EFOP-3.6.1-16-2016-00022) from the National Research, Development and Innovation Office.

ACKNOWLEDGMENTS

An earlier draft of this manuscript has been released as a pre-print at medRxiv.org (45).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2020.00465/full#supplementary-material

Table S1 | PRISMA checklist.

Table S2 | Characteristics of clinical trials including saliva as a diagnostic tool for COVID-19, registered on ClinicalTrials.gov.

Table S3 | Summary of risk-of-bias and applicability concerns in included studies.

Table S4 | Detailed summary of risk of bias and applicability across studies.

REFERENCES

1. World Health, O. *WHO Coronavirus Disease (COVID-19) Dashboard* (2020). Retrieved from: https://covid19.who.int (accessed May 05, 2020).
2. Lippi G, Simundic AM, Plebani M. Potential preanalytical and analytical vulnerabilities in the laboratory diagnosis of coronavirus disease 2019 (COVID-19). *Clin Chem Lab Med*. (2020) 58:1070–6. doi: 10.1515/cclm-2020-0285
3. World Health, O. *Laboratory Testing for Coronavirus Disease 2019 (COVID-19) in Suspected Human Cases: Interim Guidance*. Geneva (2020). Retrieved from: https://www.who.int/publications-detail/laboratory-testing-for-2019-novel-coronavirus-in-suspected-human-cases-20200117 (accessed March 19, 2020).
4. Kim YG, Yun SG, Kim MY, Park K, Cho CH, Yoon SY, et al. Comparison between saliva and nasopharyngeal swab specimens for detection of respiratory viruses by multiplex reverse transcription-PCR. *J Clin Microbiol*. (2017) 55:226–33. doi: 10.1128/JCM.01704-16
5. Li H, Liu SM, Yu XH, Tang SL, Tang CK. Coronavirus disease 2019 (COVID-19): current status and future perspectives. *Int J Antimicrobial Agents*. (2020) 55:105951. doi: 10.1016/j.ijantimicag.2020.105951
6. Sri Santosh T, Parmar R, Anand H, Srikanth K, Saritha M. A review of salivary diagnostics and its potential implication in detection of Covid-19. *Cureus*. (2020) 12:e7708. doi: 10.7759/cureus.7708

7. Varga G. Physiology of the salivary glands. *Surgery.* (2015) 33:581–6. doi: 10.1016/j.mpsur.2015.09.003
8. Dawes C, Wong DTW. Role of saliva and salivary diagnostics in the advancement of oral health. *J Dent Res.* (2019) 98:133–41. doi: 10.1177/0022034518816961
9. Humphrey SP, Williamson RT. A review of saliva: normal composition, flow, and function. *J Prosthet Dent.* (2001) 85:162–9. doi: 10.1067/mpr.2001.113778
10. Miller CS, Foley JD, Bailey AL, Campell CL, Humphries RL, Christodoulides N, et al. Current developments in salivary diagnostics. *Biomark Med.* (2010) 4:171–89. doi: 10.2217/bmm.09.68
11. Keremi B, Beck A, Fabian TK, Fabian G, Szabo G, Nagy A, et al. Stress and salivary glands. *Curr Pharm Des.* (2017) 23:4057–65. doi: 10.2174/1381612823666170215110648
12. Niedrig M, Patel P, El Wahed AA, Schadler R, Yactayo S. Find the right sample: a study on the versatility of saliva and urine samples for the diagnosis of emerging viruses. *BMC Infect Dis.* (2018) 18:707. doi: 10.1186/s12879-018-3611-x
13. Moher D, Shamseer L, Clarke M, Ghersi D, Liberati A, Petticrew M, et al. Preferred reporting items for systematic review and meta-analysis protocols (PRISMA-P) 2015 statement. *Syst Rev.* (2015) 4:1. doi: 10.1186/2046-4053-4-1
14. Azzi L, Carcano G, Gianfagna F, Grossi P, Gasperina DD, Genoni A, et al. Saliva is a reliable tool to detect SARS-CoV-2. *J Infect.* (2020) 81:e45–50. doi: 10.1016/j.jinf.2020.04.005
15. Bae S, Kim MC, Kim JY, Cha HH, Lim JS, Jung J, et al. Effectiveness of surgical and cotton masks in blocking SARS-CoV-2: a controlled comparison in 4 patients. *Ann Intern Med.* (2020) 6:M20–1342. doi: 10.7326/L20-0745
16. Fang Z, Zhang Y, Hang C, Ai J, Li S, Zhang W. Comparisons of viral shedding time of SARS-CoV-2 of different samples in ICU and non-ICU patients. *J Infect.* (2020) 81:147–78. doi: 10.1016/j.jinf.2020.03.013
17. To KK, Tsang OT, Leung WS, Tam AR, Wu TC, Lung DC, et al. Temporal profiles of viral load in posterior oropharyngeal saliva samples and serum antibody responses during infection by SARS-CoV-2: an observational cohort study. *Lancet Infect Dis.* (2020) 20:565–74. doi: 10.1016/S1473-3099(20)30196-1
18. Williams E, Bond K, Zhang B, Putland M, Williamson DA. Saliva as a non-invasive specimen for detection of SARS-CoV-2. *J Clin Microbiol.* (2020). doi: 10.1128/JCM.00776-20. [Epub ahead of print].
19. Deng Z, Hu Y, Yang P, Zheng P, Peng W, Ren B, et al. Diagnosis and treatment of an acute severe pneumonia patient with COVID-19: case report. *J Med Virol.* (2020). doi: 10.1002/jmv.25802. [Epub ahead of print].
20. Han MS, Seong MW, Heo EY, Park JH, Kim N, Shin S, et al. Sequential analysis of viral load in a neonate and her mother infected with SARS-CoV-2. *Clin Infect Dis.* (2020) 16:ciaa447. doi: 10.1093/cid/ciaa447. [Epub ahead of print].
21. Wyllie AL, Fournier J, Casanovas-Massana A, Campbell M, Tokuyama M, Vijayakumar P, et al. Saliva is more sensitive for SARS-CoV-2 detection in COVID-19 patients than nasopharyngeal swabs. *MedRxiv [preprint].* (2020). doi: 10.1101/2020.04.16.20067835
22. Whiting PF, Rutjes AW, Westwood ME, Mallett S, Deeks JJ, Reitsma JB, et al. QUADAS-2: a revised tool for the quality assessment of diagnostic accuracy studies. *Ann Intern Med.* (2011) 155:529–36. doi: 10.7326/0003-4819-155-8-201110180-00009
23. Bristol UO. *QUADAS-2. Bristol Medical School: Population Health Sciences.* (2020) Retrieved from: https://www.bristol.ac.uk/population-health-sciences/projects/quadas/quadas-2/ (accessed May 05, 2020).
24. Wilson EB. Probable inference, the law of succession, and statistical inference. *J Am Stat Assoc.* (1927) 22:209–12. doi: 10.1080/01621459.1927.10502953
25. Freeman MF, Tukey JW. Transformations related to the angular and the square root. *Ann Math Stat.* (1950) 21:607–11. doi: 10.1214/aoms/1177729756
26. DerSimonian R, Laird N. Meta-analysis in clinical trials. *Control Clin Trials.* (1986) 7:177–88. doi: 10.1016/0197-2456(86)90046-2
27. Higgins JPT, Altman DG, Gøtzsche PC, Jüni P, Moher D, Oxman AD, et al. The cochrane collaboration's tool for assessing risk of bias in randomised trials. *BMJ.* (2011) 343:d5928. doi: 10.1136/bmj.d5928
28. Cumpston M, Li T, Page MJ, Chandler J, Welch VA, Higgins JP, et al. Updated guidance for trusted systematic reviews: a new edition of the Cochrane Handbook for Systematic Reviews of Interventions. *Cochrane Database Syst Rev.* (2019) 10:Ed000142. doi: 10.1002/14651858.ED000142
29. Kaczor-Urbanowicz KE, Martin Carreras-Presas C, Aro K, Tu M, Garcia-Godoy F, Wong DT. Saliva diagnostics - current views and directions. *Exp Biol Med.* (2017) 242:459–72. doi: 10.1177/1535370216681550
30. Corstjens PL, Abrams WR, Malamud D. Detecting viruses by using salivary diagnostics. *J Am Dent Assoc.* (2012) 143(Suppl. 10):12s−18s. doi: 10.14219/jada.archive.2012.0338
31. Khurshid Z, Zafar M, Khan E, Mali M, Latif M. Human saliva can be a diagnostic tool for Zika virus detection. *J Infect Public Health.* (2019) 12:601–4. doi: 10.1016/j.jiph.2019.05.004
32. Gorchakov R, Berry RM, Patel SM, El Sahly HM, Ronca SE, Murray KO. Optimizing PCR detection of zika virus from various body fluids. *Am J Trop Med Hyg.* (2019) 100:427–33. doi: 10.4269/ajtmh.18-0755
33. Wang WK, Chen SY, Liu IJ, Chen YC, Chen HL, Yang CF, et al. Detection of SARS-associated coronavirus in throat wash and saliva in early diagnosis. *Emerg Infect Dis.* (2004) 10:1213–9. doi: 10.3201/eid1007.031113
34. Liu L, Wei Q, Alvarez X, Wang H, Du Y, Zhu H, et al. Epithelial cells lining salivary gland ducts are early target cells of severe acute respiratory syndrome coronavirus infection in the upper respiratory tracts of rhesus macaques. *J Virol.* (2011) 85:4025–30. doi: 10.1128/JVI.02292-10
35. Lu B, Huang Y, Huang L, Li B, Zheng Z, Chen Z, et al. Effect of mucosal and systemic immunization with virus-like particles of severe acute respiratory syndrome coronavirus in mice. *Immunology.* (2010) 130:254–61. doi: 10.1111/j.1365-2567.2010.03231.x
36. Silva-Boghossian CM, Colombo AP, Tanaka M, Rayo C, Xiao Y, Siqueira WL. Quantitative proteomic analysis of gingival crevicular fluid in different periodontal conditions. *PLoS ONE.* (2013) 8:e75898. doi: 10.1371/journal.pone.0075898
37. Xu H, Zhong L, Deng J, Peng J, Dan H, Zeng X, et al. High expression of ACE2 receptor of 2019-nCoV on the epithelial cells of oral mucosa. *Int J Oral Sci.* (2020) 12:8. doi: 10.1038/s41368-020-0074-x
38. Ghafouri-Fard S, Noroozi R, Vafaee R, Branicki W, Pośpiech E, Pyrc K, et al. Effects of host genetic variations on response to, susceptibility and severity of respiratory infections. *Biomed Pharmacother.* (2020) 128:110296. doi: 10.1016/j.biopha.2020.110296
39. Han P, Ivanovski S. Saliva-friend and foe in the COVID-19 outbreak. *Diagnostics.* (2020) 10:290. doi: 10.3390/diagnostics10050290
40. Zimmermann P, Curtis N. Coronavirus infections in children including COVID-19: an overview of the epidemiology, clinical features, diagnosis, treatment and prevention options in children. *Pediatr Infect Dis J.* (2020) 39:355–68. doi: 10.1097/INF.0000000000002660
41. Zou L, Ruan F, Huang M, Liang L, Huang H, Hong Z, et al. SARS-CoV-2 viral load in upper respiratory specimens of infected patients. *N Engl J Med.* (2020) 382:1177–9. doi: 10.1056/NEJMc2001737
42. Sullivan PS, Sailey C, Guest JL, Guarner J, Kelley C, Siegler AJ, et al. Detection of SARS-CoV-2 RNA and antibodies in diverse samples: protocol to validate the sufficiency of provider-observed, home-collected blood, saliva, and oropharyngeal samples. *JMIR Public Health Surveill.* (2020) 6:e19054. doi: 10.2196/19054
43. Bhattarai KR, Kim HR, Chae HJ. Compliance with saliva collection protocol in healthy volunteers: strategies for managing risk and errors. *Int J Med Sci.* (2018) 15:823–31. doi: 10.7150/ijms.25146
44. Golatowski C, Salazar MG, Dhople VM, Hammer E, Kocher T, Jehmlich N, et al. Comparative evaluation of saliva collection methods for proteome analysis. *Clin Chim Acta.* (2013) 419:42–6. doi: 10.1016/j.cca.2013.01.013
45. Czumbel LM, Kiss S, Farkas N, Mandel I, Hegyi AE, Nagy AK, et al. Saliva as a candidate for COVID-19 diagnostic testing: a meta-analysis. *medRxiv [preprint].* (2020). doi: 10.1101/2020.05.26.20112565

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

PERSPECTIVE
published: 05 August 2020
doi: 10.3389/fmicb.2020.01857

Directly Acting Antivirals for COVID-19: Where Do We Stand?

*Siew L. Teoh[1], Yi H. Lim[2], Nai M. Lai[1,3,4] and Shaun W. H. Lee[1,4,5]**

[1] School of Pharmacy, Monash University Malaysia, Subang Jaya, Malaysia, [2] School of Biosciences, Faculty of Health & Medical Sciences, Taylor's University, Subang Jaya, Malaysia, [3] School of Medicine, Faculty of Health & Medical Sciences, Taylor's University, Subang Jaya, Malaysia, [4] Asian Centre for Evidence Synthesis in Population, Implementation and Clinical Outcomes (PICO) Health and Well-being Cluster, Global Asia in the 21st Century (GA21) Platform, Monash University Malaysia, Subang Jaya, Malaysia, [5] School of Pharmacy, Faculty of Health & Medical Sciences, Taylor's University, Subang Jaya, Malaysia

The outbreak of a novel coronavirus (SARS-CoV-2) in Wuhan, China in December 2019 has now become a pandemic with no approved therapeutic agent. At the moment, the genomic structure, characteristics, and pathogenic mechanisms of SARS-CoV-2 have been reported. Based upon this information, several drugs including the directly acting antivirals have been proposed to treat people with coronavirus disease 2019 (COVID-19). This rapid review aims to describe the directly acting antivirals that have been examined for use in the management of COVID-19. Searches were conducted in three electronic databases, supplemented with a search on arXiv, bioRxiv, medRxiv, ChinaXiv, ClinicalTrials.gov, and Chinese Clinical Trial Registry for studies examining the use of antivirals in COVID-19 to identify for case reports, case series, observational studies, and randomized controlled studies describing the use of antivirals in COVID-19. Data were extracted independently and presented narratively. A total of 98 studies were included, comprising of 38 published studies and 60 registered clinical trials. These drugs include the broad spectrum antivirals such as umifenovir, protease inhibitors such as lopinavir/ritonavir as well as the RNA-dependent RNA polymerase inhibitors, remdesivir, and favipiravir. Other drugs that have been used include the nucleosidase inhibitors and polymerase acidic endonuclease inhibitors which are currently approved for prevention of influenza infections. While some of the drugs appear promising in small case series and reports, more clinical trials currently in progress are required to provide higher quality evidence.

Keywords: rapid review, systematic review, COVID-19, antivirals, pandemic

OPEN ACCESS

Edited by:
Slobodan Paessler, The University of Texas Medical Branch at Galveston, United States

Reviewed by:
Oliver Planz, University of Tübingen, Germany
Siew Pheng Lim, Denka Life Innovation Research (DLIR), Singapore

***Correspondence:**
Shaun W. H. Lee
shaun.lee@monash.edu

Specialty section:
This article was submitted to Virology, a section of the journal Frontiers in Microbiology

Received: *15 April 2020*
Accepted: *15 July 2020*
Published: *05 August 2020*

Citation:
Teoh SL, Lim YH, Lai NM and Lee SWH (2020) Directly Acting Antivirals for COVID-19: Where Do We Stand? Front. Microbiol. 11:1857. doi: 10.3389/fmicb.2020.01857

INTRODUCTION

In December 2019, an outbreak caused by a novel coronavirus was reported in Wuhan city, in Hubei province, China. The outbreak was found to be caused by a novel virus, the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (Cheng and Shan, 2020; Wu and McGoogan, 2020). Since then, the cases of SARS-CoV-2 have been reported in every single continent around the world. With over nine million individuals infected with coronavirus disease 2019 (COVID-19) and over 450 thousand death as of mid-June 2020, COVID-19 is now a public health emergency. In many individuals with COVID-19, they often present with a decrease in both $CD4^+$ and $CD8^+$ T-cells

count and suffer from acute respiratory syndrome for 7 to 10 days due to the rapid viral replication (Cheng and Shan, 2020; Zhou F. et al., 2020). Clinical features of SARS-CoV-2 infections are similar to SARS-CoV, characterized by fever, dry cough, dyspnoea or shortness of breath, diarrhea, sore throat, muscle ache, and vomiting in some patients (Meo et al., 2020; Wu and McGoogan, 2020).

The SARS-CoV-2 is a member of the family *Coronaviridae*, a positive-sense, single-stranded RNA virus that enters the mammalian cell through an interaction of viral spike glycoprotein that binds to the angiotensin-converting enzyme 2 (ACE_2) receptor (Fehr and Perlman, 2015). Following receptor binding, the virus uses the host cell receptor and endosome to enter the cell and synthesizes viral polyproteins that encode for the replicase-transcriptase complex. The virus then synthesizes RNA using its RNA-dependent RNA polymerase to synthesize structural proteins leading to completion of assembly and release of viral particles (Fehr and Perlman, 2015; Cheng and Shan, 2020). Genomic sequencing of the virus has revealed that SARS-CoV-2 has a high similarity to the bat-derived SARS-CoV, with approximately 79% identity (Wu C. et al., 2020). Studies have shown that SARS-CoV-2 is spread primarily through the respiratory system and droplets, with an incubation period of between 2 and 14 days, and a median period of 4 days (range, 2–7 days) (Livingston et al., 2020). As such, pharmacological agents that target the spike protein or host's ACE_2 proteins used to treat SARS and Middle-East Respiratory Syndrome (MERS) have been suggested as potential agents that could be used to treat patients with COVID-19. Agents proposed to eradicate the coronavirus or at least reduce the effects and hinder the contagion of the SARS-CoV-2 include repurposing currently available drugs such as monoclonal antibodies, antivirals, antimalarial among others (Fehr and Perlman, 2015; Cheng and Shan, 2020).

This intensifying outbreak has led to a surge in registered clinical trials since the infection was first reported (Zhu N. et al., 2020). In order to rapidly inform further and better design and conduct of clinical trials, there is an urgent need to provide government agencies on the investigational candidates most suitable for clinical trials. While there are major gaps in knowledge around COVID-19, especially in terms of the effectiveness and safety of various directly acting antiviral agents, a review of the characteristics of published, on-going trials and a synthesis of all available results can help inform current practice and direct future research. This rapid review was performed to provide government bodies on the evidence available in relation to the antiviral drug therapies that have been examined to date.

METHODS

Search Strategy

We performed a search of PubMed, EMBASE, Cochrane CENTRAL from inception to March 31st, 2020 to search for articles assessing the use of antivirals in patients with SARS-CoV-2 pneumonia without any language restriction. This was supplemented by a search on ClinicalTrials.gov, WHO International Clinical Trials Registry Platform and Chinese Clinical Trial Registry as well as pre-print articles on medRxiv, arXiv, bioRxiv, and ChinaXiv. Keywords used include: novel coronavirus, COVID-19, 2019-nCoV, antivirals, anti-retroviral and humans. Following peer-review, we updated our search to May 31st, 2020 on the database identified previously. We also expanded our keywords to include the following search terms: SARS-Co-v 2, abidol, tenofovir, EIDD-2801, sofosbuvir/ledipasvir, sofosbuvir/daclatasvir.

Study Selection and Data Abstraction

Articles were screened by two authors (SL, NL, and ST) independently for relevant studies. Studies which described the use of direct acting anti-viral therapies, irrespective of study designs conducted in humans were included. These could include case studies, case reports, cohort studies, observational studies or randomized controlled studies since. *In vitro*, animal studies and reviews were excluded since studies have suggested that these may not directly translate to clinical effects in human. We excluded drugs which does not act directly on virus such as antibiotics and antimalarial since these drugs have limited role in targeting the functions of the virus and preventing it from replicating in the body. All information was extracted independently by authors with discrepancies resolved thorough consensus. Due to the time constraints, the review was not registered in PROSPERO but the corresponding author can be contacted for the full protocol.

Study Quality and Reporting

The quality of all included studies which were registered and currently underway were assessed subjectively by one author, and classified anecdotally to either low, medium or high. This classification was based upon the study population > study design > sample size of trial and finally the presumed importance of results. Using this approach, a study that reports on patients would be given higher priority over those which had involved healthy subjects. In the event that the study recruited similar populations, a randomized controlled trial would be graded higher priority over a quasi-randomized study > observational study > case series > case report. Finally, a study of similar design that had reported clinical outcomes such as mortality, hospitalization days would be graded higher compared to those which had reported laboratory data only such as presence or absence of SARS-CoV-2 in patients. All data were summarized narratively due to the limited available evidence on the topic.

RESULTS

The database search identified a total of 1,416 articles of which 158 potentially relevant studies were screened. Forty-four studies were excluded based upon screening of abstract, and a further 16 were excluded since they did not include individuals with SARS-CoV-2, or were an *in vitro* studies. A total of 98 studies including nine randomized studies (RCTs) (Beigel et al., 2020; Cao et al., 2020; Chen C. et al., 2020; Goldman et al., 2020; Hung et al., 2020; Li et al., 2020; Lou et al., 2020; Wang Y. et al., 2020; Zheng F. et al., 2020) and 29 non-randomized studies (Antinori et al., 2020; Cai et al., 2020; Chen W. et al., 2020; Chen H. et al., 2020;

Deng et al., 2020; Gautret et al., 2020; Giacomelli et al., 2020; Grein et al., 2020; Haerter et al., 2020; Holshue et al., 2020; Kim et al., 2020; Lian et al., 2020; Lim et al., 2020; Liu et al., 2020; Panagopoulos et al., 2020; Shi et al., 2020; Vizcarra et al., 2020; Wang Z. et al., 2020; Xi et al., 2020; Xu et al., 2020; Yan et al., 2020; Yang et al., 2020; Ye et al., 2020; Young et al., 2020; Zhang et al., 2020; Zheng C. et al., 2020; Zhou Y. et al., 2020; Zhu Z. et al., 2020; Zuo et al., 2020) examining the use of antivirals in COVID-19 were included (**Figure 1**). We also included another 60 registered clinical trials which were at clinical phases 2, 3, or 4 (**Supplementary Appendix Table 1**, **Supplementary Appendix Figure 1**, and **Supplementary Appendix Figure 2**). Most of the trials will be mainly conducted in China but also from other countries including France, Canada, Hong Kong, Iran, Brazil, Egypt, Pakistan, Thailand, United States, Spain, and Korea. The pharmaceutical interventions found for COVID-19 treatment include remdesivir, oseltamivir, favipiravir, danoprevir, ritonavir, darunavir, baloxavir marboxil, azvudine, triazavirin, umifenovir, lopinavir either alone or in combination with other products such as human immunoglobulin, interferons, carrimycin, bevacizumab, cobicistat, and traditional Chinese medicines (see **Tables 1**, **2** for characteristics of studies identified).

Most of the registered trials were very small in size with sample size of fewer than 100 patients, with a median sample size of 145 (IQR: 60–343). In most trials, participants had to be aged 18 years and above. Most of these trials were in the recruiting stages ($n = 39$) or the preparation stages ($n = 19$). There was only limited data available on the efficacy of antivirals on COVID-19 and their clinical impact. Most of the trials will examine a myriad of primary outcomes, including time to clinical improvements,

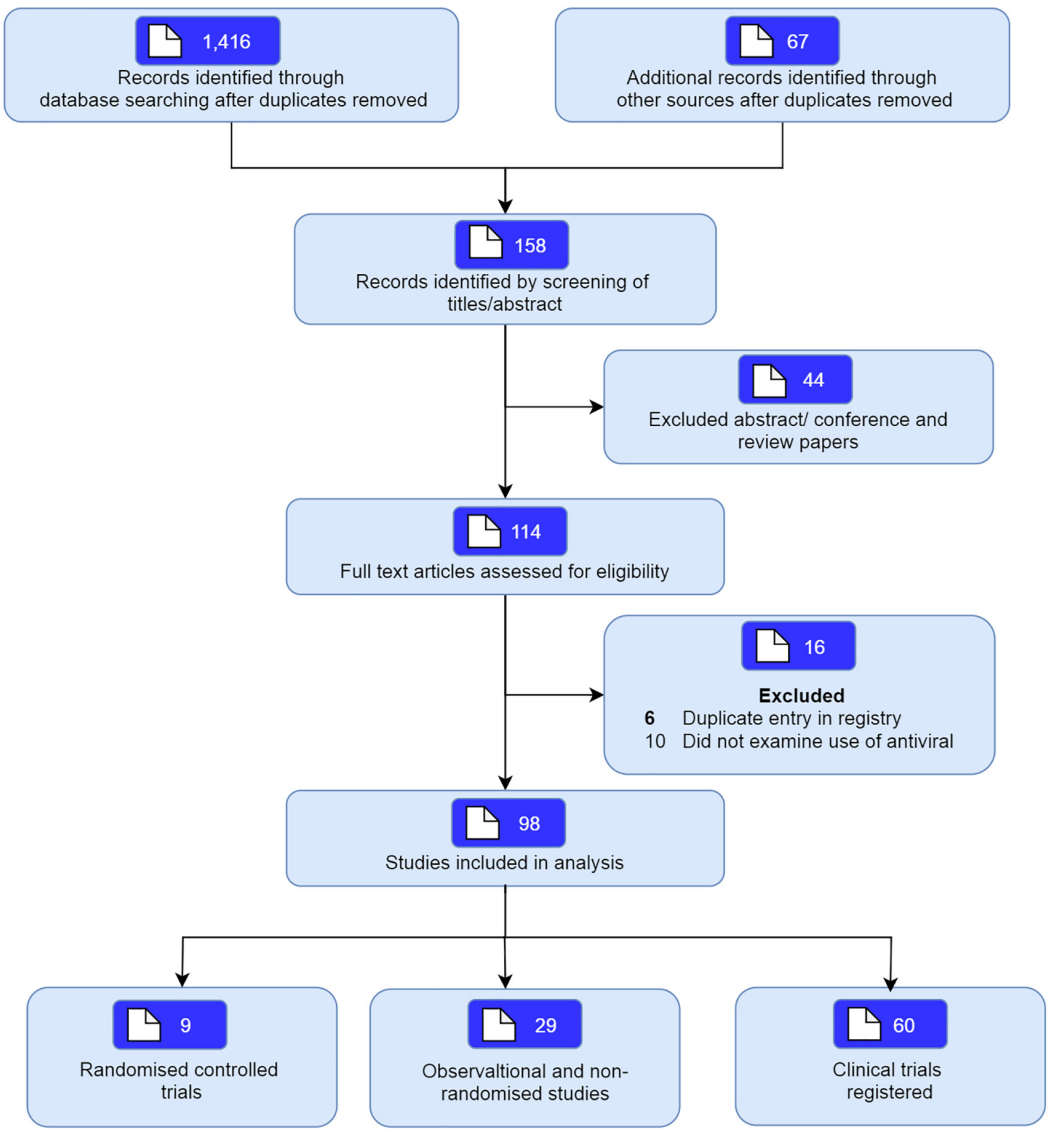


FIGURE 1 | Flow of studies searched and identified.

TABLE 1 | Study characteristics and reported outcomes from randomized controlled studies.

Study ID, Country	Study design	Disease severity	Primary outcome	Efficacy outcomes	Safety outcomes, *n* (%)
Cao et al. (2020) China	Open label single center RCT, *n* = 199 Int: Lopinavir–ritonavir (400 mg and 100 mg) twice daily for 14 days with standard care Ctr: Standard care comprising of as necessary supplemental oxygen, non-invasive and invasive ventilation, antibiotic agents, vasopressor support, renal-replacement therapy, and extracorporeal membrane oxygenation (ECMO)	Severe	Time to clinical improvement on two points (from randomization) on the following seven scale category or live discharge 1. Not hospitalized with resumption of normal activities; 2. Not hospitalized, but unable to resume normal activities 3. Hospitalized, not requiring supplemental oxygen 4. Hospitalized, requiring supplemental oxygen 5. Hospitalized, requiring nasal high-flow oxygen therapy, non-invasive mechanical ventilation, or both 6. Hospitalized, requiring ECMO, invasive mechanical ventilation, or both 7. Death	Median time to clinical improvement Int: 15 days Ctr: 16 days Hazard ratio: 1.39, 95% CI: 1.00–1.91 Mortality Int: 19 (19.2%) Ctr: 25 (25.0%) MD: −5.8%; 95% CI: −17.3 to 5.7. Median ICU length of stay Int: 6 (2 to 11) Ctr: 11 (7 to 17) MD: −5 days; 95% CI: −9 to 0 Number with clinical improvement at 14 days Int: 45 (45.5) Ctr: 30 (30.0) MD: −15.5; 95% CI: 2.2 to 28.8 Median hospital stay Int: 14 (12 to 17) Ctr: 16 (13 to 18) MD: 1, 95% CI: 0 to 2	Any adverse event (any grade), *n* (%) Int: 46 (48.4) Ctr: 49 (49.5) Any adverse event (Grade 3 or 4), *n* (%) Int: 20 (21.1) Ctr: 11 (11.1) Serious adverse event (any grade), *n* (%) Int: 19 (20.0) Ctr: 32 (32.3) Serious adverse event (Grade 3 or 4), *n* (%) Int: 17 (17.9) Ctr: 31 (31.3)
Chen C. et al. (2020) China ChiCTR2000030254	Open label multi-center RCT, *n* = 240 Int: Favipiravir 1600 mg twice daily on day 1, then 600 mg twice daily for 7–10 days with standard care Ctr: Umifenovir 200 mg three times daily for 7–10 days with standard care	Mild and moderate	Clinical recovery defined as • Normal body temperature for more than 3 days, with axillary temperature ≤ 36.6°C • Respiratory rate ≤ 24 times/min • Oxygen saturation ≥ 98% • Mild or no cough	Clinical recovery at day 7, *n* (%) Favipiravir: 71 (61.2%) Umedipavir: 62 (51.7%)	Adverse effect (all) Total patients, *n* (%) Favipiravir : 37 (31.9) Umedipavir: 28 (23.3) Total events, *n* Favipiravir : 43 Umedipavir: 33
Li et al. (2020) China NCT04252885	Open label single-centre RCT, *n* = 86 Int 1: Lopinavir–ritonavir (400 mg and 100 mg) twice daily for 7–14 days with standard care and oxygen therapy if needed Int 2: Umifenovir 200 mg three times daily for 7–14 days with standard care and oxygen therapy if needed Ctr: Standard care and oxygen therapy if needed (no antivirals)	Mild and moderate	Time to negative detection of SARS-CoV-2 nucleic acid at day 21	Mean time to negative conversion of SARS-CoV-2, mean (SD) Lopinavir/ritonavir: 9.0 (5.0) Umifenovir: 9.1 (4.4) Ctr: 9.3 (5.2) Difference between group: $p = 0.98$ Rate of positive to negative conversion at day 7, total patients, *n* (%) Lopinavir/ritonavir: 12 (35.3) Umifenovir: 13 (37.1) Ctr: 7 (41.2) Rate of positive to negative conversion at day 14, total patients, *n* (%) Lopinavir/ritonavir: 29 (85.3) Umifenovir: 32 (91.4) Ctr: 13 (76.5)	Adverse effect (all) Total patients Lopinavir/ritonavir: 12 (35.3) Umifenovir: 5 (14.3) Ctr: 0 (0) Serious adverse effect (all) Total patients Lopinavir/ritonavir: 1 (2.9) Umifenovir: 0 (0) Ctr: 0 (0)

(Continued)

TABLE 1 | Continued

Study ID, Country	Study design	Disease severity	Primary outcome	Efficacy outcomes	Safety outcomes, *n* (%)
Wang Z. et al. (2020) China NCT04257656	Multi-center RCT, *n* = 237 Int: Remdesivir 200 mg loading dose on day 1 is given, followed by 100 mg iv once-daily maintenance doses for 9 days ± concomitant use of lopinavir–ritonavir, interferons, or corticosteroids Ctr: Placebo for 10 days ± concomitant use of lopinavir–ritonavir, interferons, or corticosteroids	Severe	Time to clinical improvement within 28 days after randomization	Time to clinical improvement, median (IQR) Remdesivir: 21 (13–28) Placebo: 23 (15–28) Early symptom resolution, *n* (%) Remdesivir: 8 (11) Placebo: 7 (15) Clinical improvement rates Day 7, *n* (%) Remdesivir: 4 (3) Placebo: 2 (2) Day 14 Remdesivir: 42 (27) Placebo: 18 (23) Day 28 Remdesivir: 103 (65) Placebo: 45 (58) Duration of mechanical ventilation in days, median (IQR) Remdesivir: 7.0 (4.0–16.0) Placebo: 15.5 (6–21.0) 28 days mortality, *n* (%) Remdesivir: 22 (14) Placebo: 10 (13)	Adverse events, *n* (%) Remdesivir: 102 (66) Placebo: 50 (64) Severe adverse events, *n* (%) Remdesivir: 28 (18) Placebo: 9 (6)
Beigel et al. (2020) NCT04280705	Multi-center RCT in Europe, Asia, and America, *n* = 1,107 Int: Remdesivir 200 mg loading dose on day 1 is given, followed by 100 mg iv once-daily maintenance doses for 9 days Ctr: Placebo for 10 days	Moderate to severe	Time to recovery, defined as the first day, during the 28 days after enrolment, on which a patient satisfied categories 1, 2, or 3 on the eight-category ordinal scale.	Time to recovery, median (95% CI) Remdesivir: 11 (9–12) Placebo: 15 (13–19) No of recoveries, *n* (%) Remdesivir: 334 (63.3) Placebo: 273 (52.4) Mortality at day 14, *n* (%) Remdesivir: 32 (5.9) Placebo: 54 (10.4)	Adverse events, *n* (%) Remdesivir: 156 (28.8) Placebo: 172 (33.0) Severe adverse events, *n* (%) Remdesivir: 114 (21.1) Placebo: 141 (27.0)
Goldman et al. (2020) NCT04292899	Open label multi-center RCT in Europe, Asia and America, *n* = 397 Int: Remdesivir 200 mg loading dose on day 1 is given, followed by 100 mg iv once-daily maintenance doses for 4 days Ctr: Remdesivir 200 mg loading dose on day 1 is given, followed by 100 mg iv once-daily maintenance doses for 4 days	Moderate to severe	Clinical status on day 14, assessed on a 7-point ordinal scale on the following 1, death; 2, hospitalized, receiving invasive mechanical ventilation or ECMO; 3, hospitalized, receiving non-invasive ventilation or high-flow oxygen devices; 4, hospitalized, requiring low-flow supplemental oxygen; 5, hospitalized, not requiring supplemental oxygen but receiving ongoing medical care (related or not related to COVID-19); 6, hospitalized, requiring neither supplemental oxygen nor ongoing medical care (other than that specified in the protocol for remdesivir administration); and 7, not hospitalized	Time to clinical improvement, median Remdesivir 5 days: 10 Remdesivir 10 days: 11	Adverse events, *n* (%) Remdesivir 5 days: 141 (70) Remdesivir 10 days: 145 (74) Severe adverse events, *n* (%) Remdesivir 5 days: 42 (21) Remdesivir 10 days: 68 (35)

(Continued)

TABLE 1 | Continued

Study ID, Country	Study design	Disease severity	Primary outcome	Efficacy outcomes	Safety outcomes, *n* (%)
Hung et al. (2020) Hong Kong NCT04276688	Multicenter, open label RCT, *n* = 127 Int: Lopinavir–ritonavir (400 mg and 100 mg) twice daily, ribavirin 400 mg twice daily and three doses of eight million iu interferon beta-1b on alternate days for three doses Ctr: Lopinavir–ritonavir (400 mg and 100 mg) twice daily for 7–14 days	Unclear	Time to achieve a negative RT-PCR result for SARS-CoV-2 in a nasopharyngeal swab sample.	Time to achieve negative RT-PCR result for SARS-CoV-2, median days (IQR) Int: 7 (5–11) Ctr: 12 (8–15) Hospital stay, median days (IQR) Int: 9.0 (7.0–13.0) Ctr: 14.5 (9.3–16.0)	Adverse events, *n* (%) Int: 41 (48) Ctr: 20 (41) Severe adverse events, *n* (%) Int: 0 (0) Ctr: 1 (2)
Zheng F. et al. (2020) China ChiCTR2000029496	Open label single-center RCT, *n* = 89 Int 1: Novaferon (20 μg) twice daily Int 2: Novaferon (20 μg) twice daily + lopinavir/ritonavir (400 mg and 100 mg) twice daily Ctr: Lopinavir–ritonavir (400 mg and 100 mg) twice daily	Moderate to severe	SARS-CoV-2 clearance rates in COVID-19 patients assessed on day 6 of antiviral treatment.	SARS-CoV-2 clearance at day 3, *n* (%) Int 1: 5 (16.3) Int 2: 11 (36.7) Ctr: 3 (10.3) SARS-CoV-2 clearance at day 6, *n* (%) Int 1: 15 (50.0) Int 2: 18 (60.0) Ctr: 7 (24.1) SARS-CoV-2 clearance at day 9, *n* (%) Int 1: 17 (56.7) Int 2: 21 (70.0) Ctr: 15 (51.7) Median time to SARS-CoV-2 clearance, days Int 1: 6 Int 2: 6 Ctr: 9	Adverse events, *n* (%) Int 1: 0 (0) Int 2: 3 (10.0) Ctr: 4 (13.8) Severe adverse events, *n* (%) Int 1: 0 (0) Int 2: 0 (0) Ctr: 0 (0)
Lou et al. (2020) China ChiCTR2000029544	Open label single-center RCT, *n* = 89 Int 1: Antiviral therapy + baloxavir marboxil 80 mg daily for 4 days and on day 7 if needed Int 2: Antiviral therapy + favipiravir with loading dose of 1600 mg followed by 600 mg three times daily up to 14 days Ctr: current antiviral treatment (drug, dose and frequency not stated)	Unclear	Number of people with viral negative at day 14 Time to clinical improvement defined as 2 point improvement on a seven-category ordinal scale or live discharge from the hospital	Viral negative at day 14, *n* (%) Int 1: 7 (70) Int 2: 7 (77) Ctr: 10 (100) Time to clinical improvement median days, (IQR) Int 1: 14 (6–49) Int 2: 14 (6–38) Ctr: 14 (6–49)	Adverse events, *n* (%) Int 1: 10 (100) Int 2: 8 (88) Ctr: 9 (90) Severe adverse events, *n* (%) Int 1: 0 (0) Int 2: 0 (0) Ctr: 0 (0)

Ctr, control; Int, intervention; MD, difference; 95% CI, 95% confidence interval.

TABLE 2 | Summary of reported clinical effects on use of antivirals from non-randomized studies.

Study ID, Country	Study design	Disease severity	Efficacy outcomes	Safety outcomes, n (%)
Prospective Open-label/Cohort Study				
Antinori et al. (2020) Italy	Prospective open-label study, *n* = 35 Int: Remdesivir (compared between patients in intensive care unit and infectious diseases ward)	Severe	Intensive care unit patients: By 10 days of treatment, 4/18 (22.2%) of patients improved in hospitalization Status (1 not requiring supplemental oxygen and 3 weaned from invasive ventilation), 10/18 (55.5%) still undergoing invasive ventilation, and 4/18 (22.2%) died; By the 28 days of follow-up, 7/18 (38.9%) of patients improved in hospitalization Status (6 discharged, 1 weaned from invasive ventilation), 16.7% still undergoing mechanical ventilation and the other 44.4% died. Infectious diseases ward patients: By 10 days of treatment, 6/17 (35.3%) of patients improved in hospitalization Status (1 discharged, 3 no longer required oxygen supplementation, 2 no longer required high-flow therapy and/or non-invasive mechanical ventilation); 10 still required high-flow therapy and/or non-invasive mechanical ventilation, and 1 died. By day 28 of follow-up, hospitalization status had improved in 88.2% of the IDW patients (14 had been discharged, one no longer required oxygen supplementation) but one still required high-flow therapy and/or non-invasive mechanical ventilation.	Severe adverse advents: Hypertransaminasemia 15/35 (42.8%) Increased total bilirubin levels 7/35 (20.0%) Acute kidney injury 8/35 (22.8%) Rash 2/35 (5.7%) Any adverse event leading to treatment discontinuation 8/35 (22.8%)
Cai et al. (2020) China ChiCTR2000029600	Prospective open-label, non-randomized, *n* = 80 Int: Favipiravir 1600 mg twice daily on day 1, 600 mg twice daily from day 2–14 Ctr: Lopinavir–ritonavir (400 mg and 100 mg) twice daily for up to 14 days		Median days to viral clearance, (IQR) Int: 4 (2.5–9) Ctr: 11 (8–13) $P < 0.001$ Improvement in chest CT scans at day 14, *n* (%) Int: 32 (91.4) Ctr: 28 (62.2) $P = 0.004$	Adverse effect (all), *n* (%) Int: 4 (11.4) Ctr: 25 (55.6)
Chen W. et al. (2020) China	Prospective open-label study, *n* = 62 Int: Arbidol Ctr: Standard of care including interferon antiviral treatment	NR	Hospitalization period in the test group and control group: (16.5 ± 7.14) days and (18.55 ± 7.52) days Fever and cough in the test group were relieved markedly faster than those in the control group ($p < 0.05$); time for two consecutive negative nucleic acid tests in the test group were shorter than that in the control group.	No significant difference between the two groups for any adverse drug reaction.
Grein et al. (2020) United States, Japan, Europe, Canada	Prospective cohort study, *n* = 53 Int: Remdesivir 200 mg on day 1, then 100 mg daily for the following 9 days.	NR	Over a median follow up of 18 days (IQR 13–23) after receiving the first dose of remdesivir, 36/53 (68%) showed improvement in oxygen support, 8/53 (15%) showed worsening. By the date of most recent follow up, 25/53 (47%) had been discharged. By 28 days of follow-up, cumulative incidence of clinical improvement was 84% (95% CI 70–99). Clinical improvement was less frequent among those receiving invasive ventilation than among those receiving non-invasive oxygen support (HR 0.33; 95% CI 0.16–0.68) and among patients 70 years and older as compared to patients younger than 50 years (HR 0.29; 95% CI 0.11–0.74). 7/53 patients (13%) died after the completion of remdesivir treatment. Overall mortality from the date of admission was 0.56 per 100 hospitalization days (95% CI 0.14–0.97) and did not differ among patient receiving invasive ventilation and non-invasive oxygen support. Hazard ratio for patient receiving invasive ventilation as compared with patient receiving non-invasive oxygen support was 2.78 (95% CI 0.33–23.19). Mortality rate was higher among patients 70 years and older as compared with patients younger than 70 years (HR 11.34; 95% CI 1.36–94.17) and among those with higher serum creatinine at baseline (HR 191; 95% CI 1.22–2.99).	32 patients (60%) reported adverse events during follow up. Most common adverse events were increased hepatic enzymes, diarrhea, rash, renal impairment, and hypotension. 12 patients (23%) had serious adverse events, which all received invasive ventilation at baseline. Most common serioys adverse events were multiple organ dysfunction syndrome, septic shock, acute kidney injury, and hypotension. 4 patients (8%) discontinued remdesivir prematurely, due to worsening of pre-existing renal failure (*n* = 1), multiple organ failure (*n* = 1), elevated aminotransferases (*n* = 1), including one patient with a maculopapular rash.

(Continued)

TABLE 2 | Continued

Study ID, Country	Study design	Disease severity	Efficacy outcomes	Safety outcomes, n (%)
Retrospective cohort studies				
Deng et al. (2020) China ChiCTR2000030254	Retrospective cohort, *n* = 33 Int: Umifenovir 200 mg three times daily and lopinavir–ritonavir (400 mg and 100 mg) twice daily for 5–12 days Ctr: Lopinavir–ritonavir (400 mg and 100 mg) twice daily for 5–12 days	Moderate to severe	Negative SARS-CoV-2 detection at day 7, *n* (%) Int: 12 (75) Ctr: 6 (35) $p < 0.05$ Negative SARS-CoV-2 detection at day 14, *n* (%) Int: 15 (94) Ctr: 9 (53) $p < 0.05$ Improvement in chest CT scans at day-7 Int: 11 (69) Ctr: 5 (29) $p < 0.05$	Adverse effect (all) Total patients, *n* (%) Favipiravir : 37 (31.9) Umedipavir: 28 (23.3) Total events, *n* Favipiravir : 43 Umedipavir: 33
Giacomelli et al. (2020) Italy	Retrospective intent-to-treat analysis, *n* = 172 Lopinavir/ritonavir (LPV/r) + hydroxychloroquine (HCQ): Int: Treatment started within 5 days of symptom onset (early treatment) (25% of patients) Ctr: Treatment started later (delayed treatment) (75% of patients)		Rate of clinical improvement increased over time to 73.3% on day 30, without any significant difference between the two groups (Gray's test $P = 0.213$). No significant association between the timing of the start of treatment and the probability of 30-day mortality (adjusted odds ratio [aOR] early treatment *vs* delayed treatment = 1.45, 95% confidence interval 0.50–4.19).	8% of the patients discontinued the treatment because of severe gastrointestinal disorders attributable to LPV/r.
Kim et al. (2020) Korea	Retrospective cohort study, *n* = 65 Lopinavir–ritonavir 400 mg/100 mg twice daily (*n* = 31) Hydroxychloroquine 400 mg once daily (*n* = 34)		Median duration of treatment was 7 days Median time to negative conversion of viral RNA Lopinavir–ritonavir: 21 days Hydroxychloroquine: 28 days Lopinavir–ritonavir (aHR 2.28; 95% CI 1.24–4.21) and younger age (aHR 2.64; 95%CI 1.43–4.87) were associated with negative conversion of viral RNA. No significant difference in time to clinical improvement between lopinavir–ritonavir-treated patients and hydroxychloroquine-treated patients (median 18 days vs. 21 days).	Lymphopenia and hyperbilirubinemia were more frequent in lopinavir–ritonavir group compared with hydroxychloroquine group. One serious adverse event (ARDS) occurred in one patient treated with lopinavir–ritonavir, two serious adverse events (ARDS and shock) occurred in patients treated with hydroxychloroquine.
Lian et al. (2020) China	Retrospective cohort, *n* = 81 Int: Umifenovir 0.2 g three times a day + symptomatic treatment Ctr: symptomatic treatment	Moderate and severe	Rate of negative pharyngeal swab tests for SARS-CoV-2 within 1 week after admission: Int: 33 (73%) Ctr: 28 (78%) Time from admission to first negative test of SARS-CoV-2: Int: 6 days (4–8) Ctr: 3 days (1–7) Time from onset of symptoms to first negative test of SARS-CoV-2: Int: 18 days (12–21) Ctr: 16 days (11–21) Length of hospital stay: Int: 13 days (9–17) Ctr: 11 days (9–14)	5/45 (45%) patients in Umifenovir group and 3/36 (8%) in control group demonstrated digestive symptoms, including diarrhea and nausea.

(Continued)

TABLE 2 | Continued

Study ID, Country	Study design	Disease severity	Efficacy outcomes	Safety outcomes, n (%)
Panagopoulos et al. (2020) Greece	Retrospective cohort, *n* = 16 Group A: Lopinavir/ritonavir + hydroxychloroquine + azithromycin Group B: Hydroxychloroquine + azithromycin	NR	7/8 patients in group A recovered, one needed intubation and mechanical ventilation. 1/8 patient in group B recovered, 3/8 died, 4/8 patients needed intubation. Days of hospitalization: Group A: 14.71 ± 0.76 Group B: 11.40 ± 2.07 Days for clinical improvement (no fever): Group A: 6.00 ± 1.16 Group B: 4.4 ± 1.52 Days for negative result of RT-PCR for SARS-CoV-2: Group A: 8.86 ± 1.68 Group B: 13.8 ± 2.68	NR
Shi et al. (2020) China	Retrospective cohort study, total *n* = 184 (divided into seven groups). Symptomatic treatment group, Arbidol group, lopinavir/ritonavir group, Arbidol + lopinavir/ritonavir group, interferon group, interferon + lopinavir/ritonavir group, and interferon + darunavir group (Doses: interferon, interferon-α2β (aerosol inhalation), 100,000 U/kg, 2 times/day; Arbidol, 200 mg, 3 times/day; lopinavir/ritonavir, 2 tablets, 2 times/day; darunavir, 1 tablet, 1 time/day)	Not classified	Data extensive among seven groups, but no significant different among groups in the rates of pneumonia resolution and length of hospital stay. Pneumonia resolution after treatment, *n* (%) Int 1: 16 (53%) Int 2: 12 (44%) Int 3: 9 (36%) Int 4: 24 (59%) Int 5: 16 (76%) Int 6: 14 (61%) Ctr: 7 (41%) Length of hospital stay, mean ± SD Int 1: 15.7 days ± 6.4 Int 2: 18.4 ± 7.2 Int 3: 18.5 ± 9.5 Int 4: 16.5 ± 5.5 Int 5: 16.2 ± 7.1 Int 6: 17.4 ± 7.0 Ctr: 20.0 ± 6.0	NR
Vizcarra et al. (2020) Spain	Prospective cohort, *n* = 51 HIV-infected individuals diagnosed with COVID-19. Nine individual received protease inhibitor before COVID-19, 37 individuals received tenofovir before COVID-19 *N* = 39 HIV-infected individuals received off-label treatment for COVID-19. - Hydroxychloroquine (*n* = 30) - Azithromycin (*n* = 19) - Ritonavir/lopinavir (*n* = 14) - Tocilizumab (*n* = 4) - Systemic corticosteroids (*n* = 15)	Mild, moderate and severe	Clinical outcomes for HIV-infected COVID-19 individuals (*n* = 51): Respiratory failure, *n* (%) Mild or moderate: 4 (11%) Severe: 13 (100%) Sepsis, *n* (%) Mild or moderate: 2 (5%) Severe: 9 (69%) Critical disease or intensive care unit admission, *n* (%) Mild or moderate: 0 Severe: 6 (46%) Invasive mechanical ventilation, *n* (%) Mild or moderate: 0 Severe: 5 (38%) Death, *n* (%) Mild or moderate: 0 Severe: 2 (15%) Recovered, *n* (%) Mild or moderate: 35 (92%) Severe: 9 (69%) Duration of hospital stay, days Mild or moderate: 8 (6–17) Severe: 8 (6–19)	NR

(Continued)

TABLE 2 | Continued

Study ID, Country	Study design	Disease severity	Efficacy outcomes	Safety outcomes, n (%)
Xu et al. (2020) China	Retrospective cohort, multi-center study (n = 141). Combined group (n = 71) patients were given Arbidol and IFNa2b Monotherapy group (n = 70): patients inhaled IFNa2b for 10 to 14 days.	Mild and moderate (non-ventilated)	The median hospitalization days was 27.1 vs. 24.2 days in two group (P = 0.056). After treatment for 7 to 14 days, there was no statistically differences of the viral RNA clearance days between two groups.	There were no differences between the two groups in hemoglobin, WBC count, platelet count, ALT, AST, or creatinine during or after treatment. Thirteen patients (18.8%) treated with Arbidol demonstrated mild nausea, stomachache, but all patients could tolerate without giving up treatment.
Yan et al. (2020) China	Retrospective cohort, n = 120 Int: Lopinavir–ritonavir (400 mg and 100 mg) twice daily for 10 or more days Ctr: Standard care	Mild, moderate, severe, and critical	Median duration of treatment was 10 days (IQR: 9–10 days Median duration of SARS-CoV-2 shedding, (IQR) Int: 22 (18–29) Ctr: 28.5 (19.5–38) p = 0.02	NR
Yang et al. (2020) China	Retrospective cohort, single-center study involving frontline health professionals (n = 164), including 82 infected with COVID-19 and 82 uninfected controls. Arbidol were taken by 23.2% if the participants in the infected group and 58.5% of the participants in the uninfected group as prophylaxis against symptomatic COVID-19 requiring hospital admission.	Asymptomatic infected and uninfected groups.	The cumulative uninfected rate of health professionals in the Arbidol group was significantly higher than that of individuals in the non-Arbidol group (log-rank test, $\chi 2 = 98.74$; $P < 0.001$). Forty-eight patients (58.5%) in the infection group were hospitalized, with a median age of 39 (31–49) years, of whom 7 (14.6%) were prophylactically administered Arbidol.	NR
Ye et al. (2020) China	Retrospective cohort, n = 47. Lopinavir/Ritonavir along with Arbidol and interferon (n = 42), and "control" (no lopinavir/ritonavir, with Arbidol and interferon only, n = 5). "The per ml of LPV/r oral liquid contained 80 mg lopinavir and 20 mg ritonavir. Usage and dosage: 5 ml/time (400/100 mg) for adults, twice a day or 10 ml/time (800/200 mg) once a day with food"	Not classified	"Compared with the control group, the patients in the test group returned to normal body temperature in a shorter time (test group: 4.8 ± 1.94 days vs. control group: 7.3 ± 1.53 days, p = 0.0364)." No significant differences between groups otherwise.	The abnormal percentage of ALT and AST in the test group was lower than that in the control group.
Zheng F. et al. (2020) China	Retrospective cohort, n = 55 Mild: Intermitted low-flow oxygen therapy (≤3 L/min) and antiviral treatment for 10 days Moderate: continuous middle-flow oxygen therapy (3∼5 L/min), triple antiviral treatment, ribavirin 500 mg twice daily and recombinant interferon-α2b (5 million units) twice daily for 10 days Severe: Oxygen support including mask oxygen (>5 L/min), high flow nasal oxygen therapy (HFNO), or non-invasive ventilation (NIV), triple antiviral treatment, ribavirin and recombinant interferon-α2b (5 million units) twice daily for 10 days. All patients also received methylprednisolone (0.5∼1 mg/kg/d × 5 days). Empirical antibiotic treatment given if bacteria infection was suspected. Treatment-failure patients were prepared early for intubation and invasive mechanical ventilation and considered for ECMO	Mild, moderate and severe	Improvement in clinical symptoms, n (%) Non-severe (mild/moderate cases): 31 (91.2) Severe: 18 (85.7) p = 0.85 At least 50% improvement in chest CT scans at 7 days, n (%) Non-severe: 22 (64.7) Severe: 12 (57.4) At least 75% improvement in chest CT scans at 14 days, n (%) Non-severe: 28 (82.4) Severe: 16 (76.2) Negative SARS-CoV-2 detection, n (%) Non-severe: 33 (97.1) Severe: 20 (95.2) P = 0.92	NR

(Continued)

TABLE 2 | Continued

Study ID, Country	Study design	Disease severity	Efficacy outcomes	Safety outcomes, n (%)
Zhu Z. et al. (2020) China	Retrospective cohort, *n* = 50 Lopinavir/ritonavir group received 400 mg/100 mg twice a day for a week Umedipavir 0.2 g Arbidol three times a day	NR	Negative SARS-CoV-2 detection at day 7, *n* (%) Lopinavir/ritonavir: 8 (23.5) Umedipavir: 8 (50) Negative SARS-CoV-2 detection at day 14, *n* (%) Lopinavir/ritonavir: 19 (55.9) Umedipavir: 16 (100)	Adverse event, all, *n* (%) Lopinavir/ritonavir: 4 (11.8) Umedipavir: 6 (33.3)
Case–control				
Zhang et al. (2020) China	Case control, *n* = 190 Int: Umifenovir 200 mg three times daily for 5–10 days Ctr: Oseltamivir 75 mg once daily or placebo	NR	Number of individuals with positive COVID-19 diagnosis, *n* (%) Int: 2 (2) Ctr: 19 (21) (Odds ratio: 0.011, 95% CI: 0.001–0.125, $p = 0.003$)	NR
Zhu Z. et al. (2020) China	Case–control, *n* = 238 Int: Arbidol	Mild and severe	Median duration of SARS-CoV-2 virus shedding: 23 days (IQR, 17.8–30 days) SARS-CoV-2 RNA clearance was significantly delayed in patients who received Arbidol > 7 days after illness onset, compared with those in whom Arbidol treatment was started ≤ 7 days after illness onset (HR, 1.738 [95% CI, 1.339–2.257], $P < 0.001$).	NR
Case series				
Chen H. et al. (2020) China NCT04291729	Case series, *n* = 11 Int: Danoprevir 100 mg twice daily and ritonavir 100 mg twice daily ± with interferon-α2b atomization inhalation (5 million units) twice daily for 4–12 days with	Moderate	Use of danoprevir with ritonavir appears to be safe and effective in supressing the viral replication of SARS-CoV-2. Median days to negative SARS-CoV-2 detection, (range): 2 (1–8) Median days to improvement in chest CT scans, (range): 3 (2–4)	NR
Gautret et al. (2020) France	Case series, *n* = 80 Int: Hydroxychloroquine and azithromycin over a period of at least 3 days	Mild	Mean length of infectious disease unit stay before discharge: 5 days All patients improved clinically except one 86-year-old patient who died, and one 74-year-old patient still in intensive care. Observations: - Rapid fall of nasopharyngeal viral load was noted: 83% negative at Day 7, and 93% at Day 8. - Virus cultures from patient respiratory samples were negative: 97.5% of patients at Day 5.	Nausea or vomiting: 2.5% Diarrhea: 5.0% Blurred vision: 1.2%
Haerter et al. (2020) Germany	Case series of PLWH with COVID-19, *n* = 17 out of 33 with tenofovir use in combination with: - Bictegravir/emtricitabine (*n* = 6) - Rilpivirine/emtricitabine (*n* = 3) - Darunavir/cobicistat/emtricitabine (*n* = 3) - Elvitegravir/cobicistat/emtricitabine (*n* = 3) - Nevirapine/emtricitabine (*n* = 2)	All mild except two critical and one severe	All recovered, one death (critical)	NR
Holshue et al. (2020) United States	Case report, *n* = 1 Remdesivir (dose and frequency not reported)	NR	Improvement reported in patient condition	NR
Lim et al. (2020) South Korea	Case report, *n* = 1 Lopinavir–ritonavir (400 mg and 100 mg) twice daily for 10 or more days	Mild	Reduced viral loads and improved clinical symptoms with treatment of antiviral	NR

(Continued)

TABLE 2 | Continued

Study ID, Country	Study design	Disease severity	Efficacy outcomes	Safety outcomes, n (%)
Liu et al. (2020) China	Case series, $n = 10$ Int: Lopinavir 400 mg twice daily with interferon-α2b atomization inhalation (5 million unit) twice daily	Mild, moderate and severe	Use of lopinavir appears to be effective	Any adverse event (any grade), *n* (%) Int: 3 (30%)
Wang Z. et al. (2020) China	Case series, $n = 4$ Umifenovir 200 mg three times daily, lopinavir–ritonavir (400 mg and 100 mg) twice daily with traditional Chinese medicine for 6–15 days with supplemental oxygen	Mild, severe	Improvement reported in three patients, of which two were confirmed SARS-CoV-2 negative.	
Young et al. (2020) Singapore	Case series, $n = 18$ Int: Lopinavir–ritonavir (200 mg and 100 mg) twice daily for up to 14 days and supportive therapy ± supplemental oxygen, $n = 5$ Ctr: Supportive therapy ± supplemental oxygen	Mild and moderate	Equivocal improvements between both groups	Adverse effect (all) Int: 4 (80%)
Other study types				
Xi et al. (2020) China	Retrospective single-group study ($n = 94$). All patients were treated with Arbidol (100 mg TDS for 14 days) and moxifloxacin (0.4 g once a day for 7–14 days).	27 severe (ICU) and 57 "ordinary warded" patients	After treatment of Arbidol and moxifloxacin for 1 week, the rates of SARS-CoV-2 nucleic acid turning negative were 69.2% in the severe group and 77.8% in the ordinary group.	NR
Zuo et al. (2020) China	Retrospective cross-sectional, $n = 181$ Either lopinavir/ritonavir or lopinavir/ritonavir + IFN-α or lopinavir/ritonavir + IFN-α + Arbidol (dose, frequency not reported)	Mild, moderate and severe	Median duration of viral shedding 18.0 days (IQR 15.0–24.0) Median length of hospital stay: 17.0 days (IQR 14.0–21.0) Median time from illness onset to discharge: 23.0 days (IQR 19.0–28.5)	NR

Ctr, control; Int, intervention; NR, not reported; PLWH, people living with HIV.

number of individuals requiring mechanical ventilation, number of individuals hospitalized into ICU, length of hospitalization, mortality as well as absence of virological indicators. Three studies also used physical functioning scores based upon an ordinal 7-point scale from the WHO master protocol and the National Early Warning Score 2 (NEWS2).

DIRECT ANTIVIRALS USED IN COVID-19

Protease Inhibitors

Successful entry of the SARS-CoV-2 into the cell will depend on the activation of envelope glycoprotein by host cell protease. As such, protease enzyme inhibitors are considered an excellent drug target for patients with COVID-19 (**Table 3**). Examples of such drugs include lopinavir, ritonavir, darunavir, danoprevir and the experimental drug ASC-09. Among these agents, the most commonly examined protease inhibitor was lopinavir/ritonavir combination, using a dosing regimen of 400 mg/100 mg lopinavir/ritonavir twice daily for up to 14 days which was reported in 18 published studies (Cai et al., 2020; Cao et al., 2020; Deng et al., 2020; Giacomelli et al., 2020; Kim et al., 2020; Li et al., 2020; Lim et al., 2020; Liu et al., 2020; Shi et al., 2020; Vizcarra et al., 2020; Wang Y. et al., 2020; Yan et al., 2020; Ye et al., 2020; Young et al., 2020; Zhu Z. et al., 2020; Zuo et al., 2020). These studies were conducted in China ($n = 13$), South Korea ($n = 2$), Italy, Singapore, and Greece ($n = 1$ each). Results from the published randomized controlled studies suggest that there is limited clinical efficacy of the combination (Cao et al., 2020; Hung et al., 2020; Li et al., 2020; Zheng F. et al., 2020). In a recently completed RCT in China, the lopinavir/ritonavir combination was reported to have limited efficacy, with no difference in time to clinical improvement (median, 16 days), duration of intensive care unit stay, days of mechanical ventilation, or days of oxygen support (Cao et al., 2020). Authors reported that there appears to be some benefit when patients were given the drug therapy earlier (within 12 days of symptom onset) as they experienced a shorter time to clinical improvement (HR 1.25; 1.77–2.05 versus 1.30; 0.84–1.99). Nevertheless, given the significant drug-drug interaction and potential risk of adverse events including gastrointestinal distress such as nausea and diarrhea and hepatotoxicity, caution should be exercised while using this combination given that nearly 20% to 30% of patients have elevated transaminases at presentation (Wu and McGoogan, 2020; Wu C. et al., 2020).

While there are no RCTs on the other protease inhibitors darunavir and danoprevir, real world evidence have been reported from Germany and China (Chen H. et al., 2020; Haerter et al., 2020; Shi et al., 2020). Haerter et al. (2020) in Germany reported the outcomes of a case series of patients living with HIV treated with antiretroviral treatment including darunavir. Of the four patients treated, one died while the other three patients recovered. Shi et al. (2020) similarly reported in their case series on the limited efficacy of darunavir in terms of reducing duration from illness onset to admission and clinical symptoms. Only one small study reported the safety of danoprevir in patients with COVID-19. However, taken together these data are difficult to

TABLE 3 | Overview of mechanism of action of antivirals and recommended doses for use in COVID-19 patients.

Antiviral	Mechanism of action	Recommended dosing regimen	Contraindication	Adverse effects	Drug interactions
Broad spectrum antiviral					
Triazavirin	Guanosine nucleotide analog that inhibits RNA synthesis. The drug was developed as a potential treatment for influenza A and B, including the H5N1 strain. Triazavirin also showed activity against tick-borne encephalitis virus, forest-spring encephalitis virus in animal models.	Not available	Not available	Gastrointestinal effect	Not available
Umifenovir	Indole derivative which has dual mechanism- direct acting virucidal activity and inhibiting several stages of viral life cycle, such as virus entry, membrane fusion and viral replication. It is currently licensed in China and Russia for the prophylaxis and treatment of influenza and other respiratory viral infections. It inhibits *in vitro* hepatitis C virus, Ebola virus, Zika virus, West Nile virus, and tick-borne encephalitis virus.	200 mg every 8 h for 7 to 14 days.	Children under 2 years	Allergic reaction, gastrointestinal upset, elevated transaminases	Inducers and inhibitors of CYP3A4
RNA−dependent RNA polymerase (RdRP) inhibitor					
Favipiravir	Pyrazinecarboxamide derivative that mimics purines or purine nucleosides and selectively inhibits RNA-dependent RNA polymerase of RNA viruses during viral replication. Favipiravir showed promising *in vitro* antiviral activities against various RNA viruses, including influenza virus, West Nile virus, Ebola virus, yellow fever virus, and Chikungunya virus. It was approved in Japan in 2014 to treat novel or re-emerging pandemic influenza virus infection when other antiviral drugs are ineffective.	A higher end of the dosing range using a loading dose of 2400 mg to 3000 mg every 12 h × 2 doses followed by a maintenance dose of 1200 mg to 1800 mg every 12 h	Pregnancy, breastfeeding	Hyperuricemia, diarrhea, elevated transaminases, decreased neutrophil count, decreased appetite	CYP2C8 and aldehyde oxidase inhibitor Influenza virus vaccine (live/attenuated)
Remdesivir	Adenosine nucleotide analog and inhibitor of RNA-dependent RNA polymerase. Drug was initially developed to treat Ebola and Marburg virus infections. It has demonstrated *in vitro* and *in vivo* activity in animal models against coronaviruses including MERS and SARS.	200 mg loading dose, and 100 mg every 24 h as IV infusion.	Not recommended in patients with GFR < 30	Elevated transaminase, kidney injury, hyperglycemia, fever	Chloroquine, hydroxychloroquine
Protease inhibitor					
ASC-09 (TMC-310911)	Protease inhibitor that is structurally similar to its parent molecule, darunavir. It acts as a peptidomimetic inhibitor and dimerization inhibitor, inhibits the cleavage of polypeptides into functional proteins required for infectious HIV. It is given in combination with ritonavir.	Ritonavir/ASC-09 100 mg/300 mg twice daily	Allergic to components of ASC-09/ritonavir tablet	Fatigue, nausea, gastrointestinal effects, increase in liver enzyme level	Not available
Danoprevir	Hepatitis C virus NS3 protease inhibitor which selectively inhibits HCV replication. It is used in combination with ritonavir. Danoprevir is currently licensed in China for the treatment of chronic hepatitis C, in combination with ritonavir, peg-interferon alpha and ribavirin.	Danoprevir/ritonavir 100/100 mg twice daily	Not available	Neutropenia	Strong inhibitor of CYP3A4

(Continued)

TABLE 3 | Continued

Antiviral	Mechanism of action	Recommended dosing regimen	Contraindication	Adverse effects	Drug interactions
Darunavir	Protease inhibitor which inhibits HIV-1 protease. It selectively inhibits the cleavage of polypeptides in infected cells, thus preventing the formation of mature viral particles. It is used in combination with cobicistat or ritonavir, which are potent inhibitors of CYP3A isozymes, to increase the systemic exposure of protease inhibitor.	Darunavir/cobicistat 800 mg/150 mg once daily	Severe (Child-Pugh Class C) hepatic impairment, co-administration with CYP3A4 inhibitors	Skin rash, increased serum cholesterol, increased serum glucose, gastrointestinal effect, headache, fatigue, increased liver enzymes	Strong inhibitor and inducer of CYP3A4
Lopinavir/ritonavir	HIV protease inhibitor which selectively inhibits the cleavage of polypeptides in infected cells, thus preventing the formation of mature viral particles. Ritonavir is mainly used to enhance the action of protease inhibitor by inhibition of CYP3A4 isozymes.	400 mg/100 mg every 12 h for up to 14 days	Hypersensitivity, co-administration with CYP3A4 inducer or inhibitor	Gastrointestinal intolerance, nausea, vomiting, diarrhea, pancreatitis, hepatotoxicity, cardiac conduct abnormalities	Inducers and inhibitors of CYP3A4
Nucleoside inhibitor					
Azvudine	Azidocytidine nucleoside analog and nucleoside reverse transcriptase inhibitor. It is metabolized intracellularly into active triphosphate form and incorporates into primer strand by reverse transcriptase, resulting viral DNA chain termination. It demonstrates antiviral activity on HIV, hepatitis B virus and hepatitis C virus.	Azvudine 10 mg on day 1, then 5 mg once daily on day 2–5	Not available	Not available	Not available
Tenofovir disoproxil fumarate	Adenosine nucleotide analog and inhibitor of RNA-dependent DNA polymerase resulting in inhibition of viral replication. It is approved for treatment of Hepatitis B and HIV-1 infection.	Tenofovir disoproxil fumarate/emtricitabine 245 mg/200 mg daily	Hypersensitivity	Pruritus, increased serum lipid, gastrointestinal effect, insomnia, pain, dizziness, depression, decreased bone mineral density	Cidofovir, lopinavir/ritonavir, didanosine, atazanavir
Ribavirin	Guanosine nucleoside analog and inhibitor of virus RNA polymerase activity. It is indicated for treatment of chronic hepatitis C virus infection.	500–600 mg twice daily	Pregnancy, hemoglobinopathies, concomitant use with didanosine, CrCl < 50 mL/min	Fatigue, pyrexia, myalgia, headache, depression, hepatic decompensation	Nucleoside analogs, azathioprine
Neuroamidase inhibitor					
Oseltamivir	Potent inhibitor of influenza virus neuraminidase enzymes found on the surface of the virus, which prevents budding from the host cell, viral replication, and infectivity. It is currently licensed for used in the treatment and prophylaxis of infection with influenza viruses A (including pandemic H1N1) and influenza B.	75 mg twice daily	Hypersensitivity to oseltamivir or component of the formulation, not recommended in ESRD not undergoing dialysis	Gastrointestinal effect, headache, pain	Dichlorphenamide, probenecid, influenza virus vaccine (live/attenuated)
Polymerase acidic endonuclease inhibitors					
Baloxavir Marboxil	Selective inhibitor of influenza cap-dependent endonuclease thus preventing polymerase function and influenza virus mRNA replication. The drug is currently approved for treatment of influenza virus A and B.	80 mg on day 1, day 4 and day 7 (no more than 3 doses)	Hypersensitivity	Diarrhea, bronchitis, nausea, sinusitis, headache	Polyvalent cation-containing laxatives, antacids or oral supplements Live attenuated influenza virus

interpret given the concomitant use of drug therapies, lack of comparator treatment and heterogeneity of disease severity.

Broad Spectrum Antiviral

Another drug commonly examined is umifenovir, a broad spectrum antiviral licensed in China and Russia for influenza. Umifenovir prevents viral host cell entry by inhibiting the membrane fusion of the viral envelope and host cell cytoplasmic membrane (Blaising et al., 2014; Fink et al., 2018; Haviernik et al., 2018). The drug was suggested to have some effects in reducing the risk of COVID-19 transmission and has been examined for post-exposure prophylaxis using a dose of 200 mg orally every 8 h. In an early pilot study from China, treatment with umifenovir was found to reduce SARS-CoV-2 viral loads, with 94% of patients treated with umifenovir reported negative SARS-CoV-2 viral load compared to 53% in the control (Deng et al., 2020). Nevertheless, the results from two RCTs suggested limited efficacy in treating COVID-19 (Chen C. et al., 2020; Li et al., 2020), as the recovery rates were comparable with control.

RNA−Dependent RNA Polymerase (RdRP) Inhibitor

Favipiravir is another oral antiviral that has been examined recently. Favipiravir is a pyrazinecarboxamide derivative and guanine analog which selectively inhibits the RNA-dependent RNA polymerase (RdRP) of RNA viruses (Furuta et al., 2009). RdRP is required during the replication process of RNA viruses as it determines the replication rates and mutation of the virus to adapt to the new host environment, which ultimately influences its fidelity. As such, targeting of RdRP has become another mainstay in the treatment of SARS-CoV-2. In a pilot pre-post study in China, 80 patients with COVID-19 were treated with favipiravir with a loading dose of 1600 mg followed by a maintenance dose of 600 mg three times daily for up to 14 days. After 14 days of treatment, the authors found that patients treated with favipiravir had better treatment outcomes in terms of disease progression and viral clearance compared to those treated with lopinavir/ritonavir (Cai et al., 2020). Two recently completed RCTs in China had reported promising clinical results due to the higher 7-day recovery rates, and symptom improvements such as fever and cough (Chen C. et al., 2020; Lou et al., 2020). With no significant adverse events were reported, favipiravir is currently being examined in several clinical trials as a potential target drug for SARS-CoV-2.

Remdesivir is another nucleotide analog inhibitor of RdRP that have been extensively examined as a potential anti SARS-CoV-2 medication. The earliest report on the use of remdesivir was reported by Holshue et al. (2020), which reported improvement in the patient's condition after treatment. Since then, two RCTs on remdesivir has been conducted using a dose of remdesivir 200 mg on day 1, followed by 100 mg daily for up to 10 days. In the first RCT of 237 patients with COVID-19 by Wang Y. et al. (2020) in China, the authors found that more patients on remdesivir had clinical improvements after 28 days, and they reported faster time to symptoms improvements compared to control. Beigel et al. (2020) meanwhile reported a large multi-center RCT in Europe, Asia, and America on 1,107 patients treated with either remdesivir or placebo for 10 days. In their study, they found that the median time to recovery was much faster with remdesivir treatment, with a significantly higher number of patient who recovered. Nevertheless, there are uncertainties about the adverse effects of the drugs, and more clinical trials are underway to examine the potential of this drug in SARS-CoV-2.

Nucleosidase and Neuroamidase Inhibitors

Another class of drugs that has been used in SARS-CoV-2 is the neuroamidase inhibitors such as oseltamivir. Given that the COVID-19 outbreak in China occurred during the peak influenza season, a large proportion of patients had received oseltamivir therapy prior to the discovery of SARS-CoV-2 as these agents have been used for various influenza subtype and other RNA viruses to inhibit the spread of the influenza virus (Wang et al., 2014; Malosh et al., 2018). Several clinical trials are currently evaluating the effectiveness of oseltamivir either alone or as a combination such as with chloroquine and favipiravir, but given its pharmacological action, there is limited role of these drugs in the management of COVID-19 once influenza has been excluded.

Similarly, the neuroamidase inhibitors ribavirin and azvudine have been recommended in the initial stages for management of COVID-19, given that the symptoms were thought to be due to pneumonia. There is currently no evidence to suggest that ribavirin when used alone offers any benefit in the management of COVID-19. The combination therapy of ribavirin, lopinavir/ritonavir and interferon beta-1b was recently shown to have some positive results and would need to be explored further (Hung et al., 2020). However, as ribavirin causes a dose-dependent hematological toxicity, and is a known teratogen, there is limited value of this drug in the treatment of COVID-19.

Polymerase Acidic Endonuclease Inhibitors

The only drug in its class examined identified in the current review was baloxavir marboxil. This drug targets the viral polymerase acidic protein to block the endonuclease function, resulting in the inhibition of virus mRNA transcription and infection (Koszalka et al., 2019; Locke et al., 2019). Only one small clinical study in China has been identified in the current review, but due to the small sample the implications will be limited (ChiCTR2000029548).

DISCUSSION

With no therapeutic agent is currently known to be effective for COVID-19, multiple different antivirals have been examined based upon the early *in vitro* evidence against SARS-CoV. While several case series and reports showed improvements with use of lopinavir-ritonavir, the recently published study by Cao et al. (2020) have showed limited benefits highlighting the difficulty in

finding an appropriate agents for rapid implementation in such outbreaks. It remains unfortunate that this therapy is ineffective, given that this would have represented an immediate and safe oral therapy for COVID-19. For most of the current trials reported, these are underpowered and unlikely to provide the healthcare community with the necessary high quality evidences needed to combat this pandemic if taken individually. In addition, most of the trials registered will only include patients aged 18 and above, and thus will unlike to provide the necessary information on children, adolescents, pregnant women or even those with respiratory diseases (Lee, 2020).

These trials also included a wide range of primary outcomes including time to clinical improvements, number of individuals requiring mechanical ventilation, number of individuals hospitalized into ICU, length of hospitalization, mortality as well as absence of virological indicators. As most of the outcomes that will be reported varied, and will include subjective outcomes, this may lead to measurement bias. Importantly, few of the current trials have reported on mortality in their study either as a primary or secondary outcome. While the case fatality rates differs between countries, ranging from as low as 0.3% to as high as 11.0%, these reports have not been forthcoming in all the included studies and should be given attention (Rajgor et al., 2020). In addition, most of the current studies are not coordinated, leading to inconsistencies among trials in their definitions of conditions and inclusion criteria, the design and delivery of intervention and comparison, as well as measurement of the outcomes. Cognisant of this, the World Health Organization (WHO) is initiating a clinical trials experts group which will aim to develop a master protocol for a RCT to evaluate efficacy of therapeutics against nCov (World Health Organization, 2020). Other impending initiatives include the strengthening of management and coordination of the promising drugs such as remdesivir and favipiravir, which should be prioritized for clinical studies. This is based upon the potential activity of both agents against RNA polymerase, established use in novel influence and also oral bioavailability. This ideally should involve the pharmacist who can help in the development of treatment protocols, monitoring of drug adverse events as well as assist in the expanded access of these new investigational drugs (Lee et al., 2019; Stevens et al., 2020).

Investigators should also consider using other clinical trial designs including step-wedge design which may reduce the need for large sample sizes (Baio et al., 2015). In addition, a database should also be setup to share all available existing data between sites and countries, which effectively create a real-world evidence study network, which can increase the speed of information dissemination especially in pandemics such as COVID-19. Indeed, there is a need for researchers to report as much details as possible to ensure reproducibility of results especially as these studies currently use very weak outcomes which can limit the efficacy assessment. Nevertheless, the development of clinical trials during an outbreak is an adaptive process, with new evidence being generated at an impressive rate. As such, we believe that these results generated will inform the adaptation of existing and new trials that are being developed. Indeed, with progressive release of trial results, there is a need for a living systematic review to progressively update the pooled results with each additional trial included. This is crucial in view of the small sample size of individual studies.

Nevertheless, we acknowledge that there are some limitations to this review, given that we had only one reviewer who had conducted the search. In addition, this review also included several pre-print articles which have not been peer-reviewed and thus may not provide the academic rigor normally required for published studies. However, in view of the evolving situation of COVID-19 and the need for rapid understanding of this disease, the decision to include these studies were needed in order for us to provide the readers with the most updated information available. Most of the published treatment data to date are derived from observational studies which have relatively small sample size, which may introduce risk regarding the magnitude of effect sizes.

In summary, this updated review of antivirals in COVID-19 showed that there is limited information available to guide clinical practice as well as the need for a more coordinated research network to seek the best therapeutic options especially in pandemics. While several agents reviewed have suggested some potential benefits of therapy, the evidence remains inconclusive.

AUTHOR CONTRIBUTIONS

SL conceived the study, conducted the analysis, and wrote the draft. ST, YL, and NL collected the data, conducted the analysis, and edited the draft. All authors approved the final draft.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.01857/full#supplementary-material

REFERENCES

Antinori, S., Cossu, M. V., Ridolfo, A. L., Rech, R., Bonazzetti, C., Pagani, G., et al. (2020). Compassionate remdesivir treatment of severe Covid-19 pneumonia in intensive care unit (ICU) and Non-ICU patients: clinical outcome and differences in post-treatment hospitalisation status. *Pharmacol. Res.* 158:104899. doi: 10.1016/j.phrs.2020.104899

Baio, G., Copas, A., Ambler, G., Hargreaves, J., Beard, E., and Omar, R. Z. (2015). Sample size calculation for a stepped wedge trial. *Trials* 16:354.

Beigel, J. H., Tomashek, K. M., Dodd, L. E., Mehta, A. K., Zingman, B. S., Kalil, A. C., et al. (2020). Remdesivir for the treatment of Covid-19 — Preliminary report. *N. Engl. J. Med.* doi: 10.1056/NEJMc2022236 [Epub ahead of print].

Blaising, J., Polyak, S. J., and Pécheur, E. I. (2014). Arbidol as a broad-spectrum antiviral: an update. *Antiviral Res.* 107, 84–94. doi: 10.1016/j.antiviral.2014.04.006

Cai, Q., Yang, M., Liu, D., Chen, J., Shu, D., Xia, J., et al. (2020). Experimental treatment with favipiravir for COVID-19: an open-label control study. *Engineering* doi: 10.1016/j.eng.2020.03.007 [Epub ahead of print].

Cao, B., Wang, Y., Wen, D., Liu, W., Wang, J., Fan, G., et al. (2020). A trial of lopinavir–ritonavir in adults hospitalized with severe Covid-19. *N. Engl. J. Med.* 382, 1787–1799.

Chen, C., Huang, J., Cheng, Z., Wu, J., Chen, S., Zhang, Y., et al. (2020). Favipiravir versus arbidol for COVID-19: a randomized clinical trial. *medRxiv* [Preprint]. doi: 10.1101/2020.03.17.20037432.t

Chen, W., Yao, M., Fang, Z., Lv, X., Deng, M., and Wu, Z. (2020). A study on clinical effect of arbidol combined with adjuvant therapy on COVID-19. *J. Med. Virol.* doi: 10.1002/jmv.26142 [Epub ahead of print].

Chen, H., Zhang, Z., Wang, L., Huang, Z., Gong, F., Li, X., et al. (2020). First clinical study using HCV protease inhibitor danoprevir to treat naïve and experienced COVID-19 patients. *medRxiv* [Preprint]. doi: 10.1101/2020.03.22.20034041

Cheng, Z. J., and Shan, J. (2020). 2019 Novel coronavirus: where we are and what we know. *Infection* 48, 155–163. doi: 10.1007/s15010-020-01401-y

Deng, L., Li, C., Zeng, Q., Liu, X., Li, X., Zhang, H., et al. (2020). Arbidol combined with LPV/r versus LPV/r alone against Corona Virus Disease 2019: a retrospective cohort study. *J. Infect.* 81, e1–e5. doi: 10.1016/j.jinf.2020.03.002

Fehr, A. R., and Perlman, S. (2015). Coronaviruses: an overview of their replication and pathogenesis. *Methods Mol. Biol.* 1282, 1–23. doi: 10.1007/978-1-4939-2438-7_1

Fink, S. L., Vojtech, L., Wagoner, J., Slivinski, N. S. J., Jackson, K. J., Wang, R., et al. (2018). The antiviral drug arbidol inhibits Zika Virus. *Sci. Rep.* 8:8989.

Furuta, Y., Takahashi, K., Shiraki, K., Sakamoto, K., Smee, D. F., Barnard, D. L., et al. (2009). T-705 (favipiravir) and related compounds: novel broad-spectrum inhibitors of RNA viral infections. *Antiviral Res.* 82, 95–102. doi: 10.1016/j.antiviral.2009.02.198

Gautret, P., Lagier, J.-C., Parola, P., Hoang, V. T., Meddeb, L., Sevestre, J., et al. (2020). Clinical and microbiological effect of a combination of hydroxychloroquine and azithromycin in 80 COVID-19 patients with at least a six-day follow up: a pilot observational study. *Travel Med. Infect. Dis.* 34:101663. doi: 10.1016/j.tmaid.2020.101663

Giacomelli, A., Pagani, G., Ridolfo, A. L., Oreni, A., Conti, F., Pezzati, L., et al. (2020). Early administration of lopinavir/ritonavir plus hydroxychloroquine does not alter the clinical course of SARS-CoV-2 infection: a retrospective cohort study. *medRxiv* [Preprint]. doi: 10.1101/2020.06.05.20123299

Goldman, J. D., Lye, D. C. B., Hui, D. S., Marks, K. M., Bruno, R., Montejano, R., et al. (2020). Remdesivir for 5 or 10 Days in Patients with Severe Covid-19. *N. Engl. J. Med.* doi: 10.1056/NEJMoa2015301 [Epub ahead of print].

Grein, J., Ohmagari, N., Shin, D., Diaz, G., Asperges, E., Castagna, A., et al. (2020). Compassionate use of remdesivir for patients with severe Covid-19. *N. Engl. J. Med.* 382, 2327–2336.

Haerter, G., Spinner, C. D., Roider, J., Bickel, M., Krznaric, I., Grunwald, S., et al. (2020). COVID-19 in people living with human immunodeficiency virus: a case series of 33 patients. *Infection* 1–6. doi: 10.1101/2020.07.11.20151688

Haviernik, J., Štefánik, M., Fojtíková, M., Kali, S., Tordo, N., Rudolf, I., et al. (2018). Arbidol (Umifenovir): a broad-spectrum antiviral drug that inhibits medically important arthropod-borne flaviviruses. *Viruses* 10:184. doi: 10.3390/v10040184

Holshue, M. L., Debolt, C., Lindquist, S., Lofy, K. H., Wiesman, J., Bruce, H., et al. (2020). First Case of 2019 novel coronavirus in the United States. *N. Engl. J. Med.* 382, 929–936.

Hung, I. F.-N., Lung, K.-C., Tso, E. Y.-K., Liu, R., Chung, T. W.-H., Chu, M.-Y., et al. (2020). Triple combination of interferon beta-1b, lopinavir & ritonavir, and ribavirin in the treatment of patients admitted to hospital with COVID-19: an open-label, randomised, phase 2 trial. *Lancet* 395, 1695–1704.

Kim, J.-W., Kim, E. J., Kwon, H. H., Jung, C. Y., Kim, K. C., Choe, J.-Y., et al. (2020). Lopinavir-ritonavir versus hydroxychloroquine for viral clearance and clinical improvement in patients with mild to moderate coronavirus disease 2019. *Korean J. Intern. Med.* doi: 10.3904/kjim.2020.224 [Epub ahead of print].

Koszalka, P., Tilmanis, D., Roe, M., Vijaykrishna, D., and Hurt, A. C. (2019). Baloxavir marboxil susceptibility of influenza viruses from the Asia-Pacific, 2012–2018. *Antiviral Res.* 164, 91–96. doi: 10.1016/j.antiviral.2019.02.007

Lee, S., Mak, V., and Tang, Y. (2019). Pharmacist services in nursing home: a systematic review and meta-analysis. *Br. J. Clin. Pharmacol.* 85, 2668–2688.

Lee, S. W. H. (2020). Coronavirus (COVID-19): what the tuberculosis (TB) community can learn. *Prog. Drug Discov. Biomed. Sci.* 3:a0000090. doi: 10.3687/pmmb.a0000090

Li, Y., Xie, Z., Lin, W., Cai, W., Wen, C., Guan, Y., et al. (2020). An exploratory randomized, controlled study on the efficacy and safety of lopinavir/ritonavir or arbidol treating adult patients hospitalized with mild/moderate COVID-19 (ELACOI). *medRxiv* [Preprint]. doi: 10.1101/2020.03.19.20038984

Lian, N., Xie, H., Lin, S., Huang, J., Zhao, J., and Lin, Q. (2020). Umifenovir treatment is not associated with improved outcomes in patients with coronavirus disease 2019: a retrospective study. *Clin. Microbiol. Infect.* 26, 917–921. doi: 10.1016/j.cmi.2020.04.026

Lim, J., Jeon, S., Shin, H.-Y., Kim, M. J., Seong, Y. M., Lee, W. J., et al. (2020). Case of the index patient who caused tertiary transmission of COVID-19 infection in Korea: the application of lopinavir/ritonavir for the treatment of COVID-19 infected pneumonia monitored by quantitative RT-PCR. *J. Korean Med. Sci.* 35:e79.

Liu, F., Xu, A., Zhang, Y., Xuan, W., Yan, T., Pan, K., et al. (2020). Patients of COVID-19 may benefit from sustained lopinavir-combined regimen and the increase of eosinophil may predict the outcome of COVID-19 progression. *Int. J. Infect. Dis.* 95, 183–191. doi: 10.1016/j.ijid.2020.03.013

Livingston, E., Bucher, K., and Rekito, A. (2020). Coronavirus Disease 2019 and Influenza 2019-2020. *JAMA* 323, 1122–1122.

Locke, S. C., Splawn, L. M., and Cho, J. C. (2019). Baloxavir marboxil: a novel cap-dependent endonuclease (CEN) inhibitor for the treatment of acute uncomplicated influenza. *Drugs Today* 55, 359–366.

Lou, Y., Liu, L., and Qiu, Y. (2020). Clinical outcomes and plasma concentrations of baloxavir marboxil and favipiravir in COVID-19 patients: an exploratory randomized, controlled trial. *medRxiv* [Preprint].

Malosh, R. E., Martin, E. T., Heikkinen, T., Brooks, W. A., Whitley, R. J., and Monto, A. S. (2018). Efficacy and safety of oseltamivir in children: systematic review and individual patient data meta-analysis of randomized controlled trials. *Clin. Infect. Dis.* 66, 1492–1500. doi: 10.1093/cid/cix1040

Meo, S., Alhowikan, A., Al-Khlaiwi, T., Meo, I., Halepoto, D., Iqbal, M., et al. (2020). Novel coronavirus 2019-nCoV: prevalence, biological and clinical characteristics comparison with SARS-CoV and MERS-CoV. *Eur. Rev. Med. Pharmacol. Sci.* 24, 2012–2019.

Panagopoulos, P., Petrakis, V., Panopoulou, M., Trypsianis, G., Penlioglou, T., Pnevmatikos, I., et al. (2020). Lopinavir/ritonavir as a third agent in the antiviral regimen for SARS-CoV-2 infection. *J. Chemother.* 1–5. doi: 10.1080/1120009x.2020.1775424 [Epub ahead of print].

Rajgor, D. D., Lee, M. H., Archuleta, S., Bagdasarian, N., and Quek, S. C. (2020). The many estimates of the COVID-19 case fatality rate. *Lancet Infect. Dis.* 20, 776–777. doi: 10.1016/s1473-3099(20)30244-9

Shi, X., Lu, Y., Li, R., Tang, Y., Shi, N., Song, F., et al. (2020). Evaluation of antiviral therapies for coronavirus disease 2019 pneumonia in Shanghai, China. *J. Med. Virol.* 16:10.1002/jmv.25893.

Stevens, M. P., Patel, P. K., and Nori, P. (2020). Involving antimicrobial stewardship programs in COVID-19 response efforts: all hands on deck. *Infect. Control Hosp. Epidemiol.* 41, 744–745. doi: 10.1017/ice.2020.69

Vizcarra, P., Pérez-Elías, M. J., Quereda, C., Moreno, A., Vivancos, M. J., Dronda, F., et al. (2020). Description of COVID-19 in HIV-infected individuals: a single-centre, prospective cohort. *Lancet HIV* S2352-3018, 30164–30168.

Wang, R. R., Yang, Q. H., Luo, R. H., Peng, Y. M., Dai, S. X., Zhang, X. J., et al. (2014). Azvudine, a novel nucleoside reverse transcriptase inhibitor showed good drug combination features and better inhibition on drug-resistant strains than lamivudine in vitro. *PLoS One* 9:e105617. doi: 10.1371/journal.pone.0105617

Wang, Y., Zhang, D., Du, G., Du, R., Zhao, J., Jin, Y., et al. (2020). Remdesivir in adults with severe COVID-19: a randomised, double-blind, placebo-controlled, multicentre trial. *Lancet* 395, 1569–1578.

Wang, Z., Chen, X., Lu, Y., Chen, F., and Zhang, W. (2020). Clinical characteristics and therapeutic procedure for four cases with 2019 novel coronavirus pneumonia receiving combined Chinese and Western medicine treatment. *Bio Sci. Trends* 14, 64–68. doi: 10.5582/bst.2020.01030

World Health Organization (2020). *WHO R&D Blueprint - Novel Coronavirus COVID-19 Therapeutic Trial Synopsis*. Geneva: World Health Organization.

Wu, C., Chen, X., Cai, Y., Zhou, X., Xu, S., Huang, H., et al. (2020). Risk factors associated with acute respiratory distress syndrome and death in patients with coronavirus disease 2019 pneumonia in Wuhan, China. *JAMA Int. Med.* 180, 1–11.

Wu, F., Zhao, S., Yu, B., Chen, Y.-M., Wang, W., Song, Z.-G., et al. (2020). A new coronavirus associated with human respiratory disease in China. *Nature* 579, 265–269.

Wu, Z., and McGoogan, J. M. (2020). Characteristics of and important lessons from the coronavirus disease 2019 (COVID-19) outbreak in China: summary of a report of 72 314 cases from the Chinese Center for Disease Control and Prevention. *JAMA* 323, 1239–1242.

Xi, W.-N., Jin, D., Sun, K., Yu, R., Yao, X. B., Zou, B.-S., et al. (2020). Treatment with arbidol and moxifloxacin in ordinary and severe adult patients infected with COVID-19. *MedRxiv* [Preprint]. doi: 10.1101/2020.05.30.20117598

Xu, P., Huang, J., Fan, Z., Huang, W., Qi, M., Lin, X., et al. (2020). Arbidol/IFN-α2b therapy for patients with corona virus disease 2019: a retrospective multicenter cohort study. *Microbes Infect.* 22, 200–205. doi: 10.1016/j.micinf.2020.05.012

Yan, D., Liu, X.-Y., Zhu, Y.-N., Huang, L., Dan, B.-T., Zhang, G.-J., et al. (2020). Factors associated with prolonged viral shedding and impact of Lopinavir/Ritonavir treatment in hospitalised non-critically ill patients with SARS-CoV-2 infection. *Eur. Respirat. J.* 56:2000799. doi: 10.1183/13993003.00799-2020

Yang, C., Ke, C., Yue, D., Li, W., Hu, Z., Liu, W., et al. (2020). Effectiveness of arbidol for COVID-19 prevention in health professionals. *Front. Public Health* 8:249. doi: 10.3389/fpubh.2020.00249

Ye, X., Luo, Y., Xia, S., Sun, Q., Ding, J., Zhou, Y., et al. (2020). Clinical efficacy of lopinavir/ritonavir in the treatment of coronavirus disease 2019. *Eur. Rev. Med. Pharmacol. Sci.* 24, 3390–3396.

Young, B. E., Ong, S. W. X., Kalimuddin, S., Low, J. G., Tan, S. Y., Loh, J., et al. (2020). Epidemiologic features and clinical course of patients infected with SARS-CoV-2 in Singapore. *JAMA* 323, 1488–1494.

Zhang, J.-N., Wang, W.-J., Peng, B., Peng, W., Zhang, Y.-S., Wang, Y.-L., et al. (2020). Potential of arbidol for post-exposure prophylaxis of COVID-19 transmission-a preliminary report of a retrospective cohort study. *Curr. Med. Sci.* 30, 1–6.

Zheng, C., Wang, J., Guo, H., Lu, Z., Ma, Y., Zhu, Y., et al. (2020). Risk-adapted treatment strategy for COVID-19 patients. *Int. J. Infect. Dis.* 94, 74–77. doi: 10.1016/j.ijid.2020.03.047

Zheng, F., Zhou, Y., Zhou, Z., Ye, F., Huang, B., Huang, Y., et al. (2020). A novel protein drug, novaferon as the potential antiviral drug for COVID-19. *medRxiv* [Preprint]. doi: 10.1101/2020.04.24.20077735

Zhou, F., Yu, T., Du, R., Fan, G., Liu, Y., Liu, Z., et al. (2020). Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: a retrospective cohort study. *Lancet* 395, 1054–1062. doi: 10.1016/s0140-6736(20)30566-3

Zhou, Y., He, X., Zhang, J., Xue, Y. E., Liang, M., Yang, B., et al. (2020). Prolonged SARS-CoV-2 viral shedding in patients with COVID-19 was associated with delayed initiation of arbidol treatment: a retrospective cohort study. *medRxiv* [Preprint]. doi: 10.1101/2020.06.09.20076646

Zhu, N., Zhang, D., Wang, W., Li, X., Yang, B., Song, J., et al. (2020). A novel coronavirus from patients with pneumonia in China, 2019. *N. Engl. J. Med.* 382, 727–733.

Zhu, Z., Lu, Z., Xu, T., Chen, C., Yang, G., Zha, T., et al. (2020). Arbidol monotherapy is superior to lopinavir/ritonavir in treating COVID-19. *J. Infect.* 81, e21–e23. doi: 10.1016/j.jinf.2020.03.060

Zuo, Y., Liu, Y., Zhong, Q., Zhang, K., Xu, Y., and Wang, Z. (2020). Lopinavir/ritonavir and interferon combination therapy may help shorten the duration of viral shedding in patients with COVID-19: a retrospective study in two designated hospitals in Anhui, China. *J. Med. Virol.* doi: 10.1002/jmv.26127 [Epub ahead of print].

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pediatrics

BRIEF RESEARCH REPORT
published: 05 August 2020
doi: 10.3389/fped.2020.00453

Quantitative Assessment of Parenchymal Involvement Using 3D Lung Model in Adolescent With Covid-19 Interstitial Pneumonia

Luca Borro[1]*, *Paolo Ciliberti*[2], *Teresa Pia Santangelo*[1], *Andrea Magistrelli*[1], *Andrea Campana*[3], *Francesca Calò Carducci*[4], *Marabotto Caterina*[5], *Paolo Tomà*[1] and *Aurelio Secinaro*[1]

[1] Department of Imaging, Bambino Gesù Children's Hospital IRCSS, Rome, Italy, [2] Pediatric Cardiology and Pediatric Cardiac Surgery Department, Bambino Gesù Children's Hospital IRCSS, Rome, Italy, [3] Department of Pediatric Medicine, Bambino Gesù Children's Hospital, Rome, Italy, [4] Pediatric University Hospital-Department, Bambino Gesù Children's Hospital, Rome, Italy, [5] Unit of General Pediatrics and Pediatric Infectious Diseases, Bambino Gesù Children's Hospital, Rome, Italy

Background: Amount of parenchymal involvement in patients with interstitial pneumonia Covid-19 related, seems to be associated with a worse prognosis. Nowadays 3D reconstruction imaging is expanding its role in clinical medical practice. We aimed to use 3D lung reconstruction of a young lady affected by Sars-CoV2 infection and interstitial pneumonia, to better visualize, and quantitatively assess the parenchymal involvement.

Methods: Volumetric Chest CT scan was performed in a 15 years old girl with interstitial lung pneumonia, Sars-CoV2 infection related. 3D modeling of the lungs, with differentiation of healthy and affected parenchymal area were obtained by using multiple software.

Results: 3D reconstruction imaging allowed us to quantify the lung parenchyma involved, Self-explaining 3D images, useful for the understanding, and discussion of the clinical case were also obtained.

Conclusions: Quantitative Assessment of Parenchymal Involvement Using 3D Lung Model in Covid-19 Infection is feasible and it provides information which could play a role in the management and risk stratification of these patients.

Keywords: 3D lung reconstructions, 3D modeling in pneumonia, 3D parenchima reconstruction, 3D rendering in pneumonia, 3D in Covid19, 3D quantify in pneumonia

OPEN ACCESS

Edited by:
Silvia Schievano, University College London, United Kingdom

Reviewed by:
Giovanni Biglino, University of Bristol, United Kingdom
Kristian Mortensen, Great Ormond Street Hospital for Children NHS Foundation Trust, United Kingdom

***Correspondence:**
Luca Borro
luca.borro@opbg.net

Specialty section:
This article was submitted to Pediatric Infectious Diseases, a section of the journal Frontiers in Pediatrics

Received: *07 April 2020*
Accepted: *30 June 2020*
Published: *05 August 2020*

Citation:
Borro L, Ciliberti P, Santangelo TP, Magistrelli A, Campana A, Carducci FC, Caterina M, Tomà P and Secinaro A (2020) Quantitative Assessment of Parenchymal Involvement Using 3D Lung Model in Adolescent With Covid-19 Interstitial Pneumonia. Front. Pediatr. 8:453. doi: 10.3389/fped.2020.00453

INTRODUCTION

The amount of parenchymal involvement in patients with interstitial pneumonia Covid-19 related seems to be associated with a worse prognosis (1).

Nowadays 3D reconstruction imaging is expanding its role in clinical medicine (2–5).

We describe the use of 3D lung reconstruction of a young lady affected by Covid-19 infection and interstitial pneumonia, to quantitatively assess the lung parenchyma involved.

In March, 2020, a 15-year-old girl was admitted to our Emergency Department, with a 7-day history of fever and anosmia. She had been complaining exertional shortness of breath over the previous 2 days. At initial evaluation oxygen saturation was 97% in room air. Chest X-Ray showed no significant consolidation with perihilar infiltrates mainly on the left basal side.

Her pharyngeal swab Real-Time Polymerase Chain Reaction (RT-PCR) was positive for Sars-Cov2 infection.

As further investigation the patient underwent Chest-CT. Non-contrast spiral high isotropic resolution CT acquisition was performed. In the left lower lobe multiple shaded areas of increased density, Ground Glass Opacity (GGO) type, were observed. There were also subpleural parenchymal "rounded" consolidations. Similar changes were seen in the lingular segment. The CT pattern was reliably related to a viral pneumonia with exclusive left lung involvement (6). Experimental treatment with azithromycin and hydroxychloroquine was started, with progressive improvement of the clinical condition.

Using CT images we performed a 3D advanced segmentation and reconstruction of both lungs, in order to better delineate and quantitatively assess the amount of parenchymal abnormalities.

Written informed consent of the family, for use of imaging and clinical data for research purposes, was obtained.

METHODS

CT high spatial resolution images with an isotropic voxel size of 1 mm and a smooth filter were used for the segmentation. The dataset was uploaded on an advanced 3D post-processing platform "Mimics" (Materialize, Belgium) and three different segmentation masks were defined: parenchyma, GGO areas, and spot consolidations. Ground-glass opacity (GGO) has been defined as an area of blurry increased lung opacity where vessels and bronchial structures may still be seen, whilst in consolidation such structures are concealed. Most commonly, GGO areas suggest inflammatory, or infiltrative interstitial lung disorders, such as interstitial pneumonia. Consolidation conversely is a region of normally compressible lung tissue that has filled with liquid instead of air, and suggest an infiltrative or inflammatory process involving the entire lung tissue.

Two semi-automatic reconstruction methods were applied. In particular, the thresholding algorithm, was an effective tool to segment normal lung parenchyma (7) (**Figure 1A**), whilst the region-growing algorithm was used for ground glass areas (**Figure 1B**) and spot consolidations (**Figure 1C**). In addition, some small areas of consolidation have been manually segmented.

We measured density values on healthy right lung with Hounsfield values from −1024Hu to −543Hu similar to typical range values of pediatric lungs (8).

Both GGO and parenchymal consolidations Hounsfield ranges were obtained by making several measurements on a different CT Slices (−957Hu to +1246Hu for GGO areas, and −834Hu to +356Hu for consolidations areas). Any transition areas between the two masks have been optimized with Boolean difference operations aimed at better defining the consolidations and GGO boundaries.

Each of the three masks has been 3D reconstructed with a specific mesh quality setup to obtain the highest resolution and the best 3D-2D correspondence.

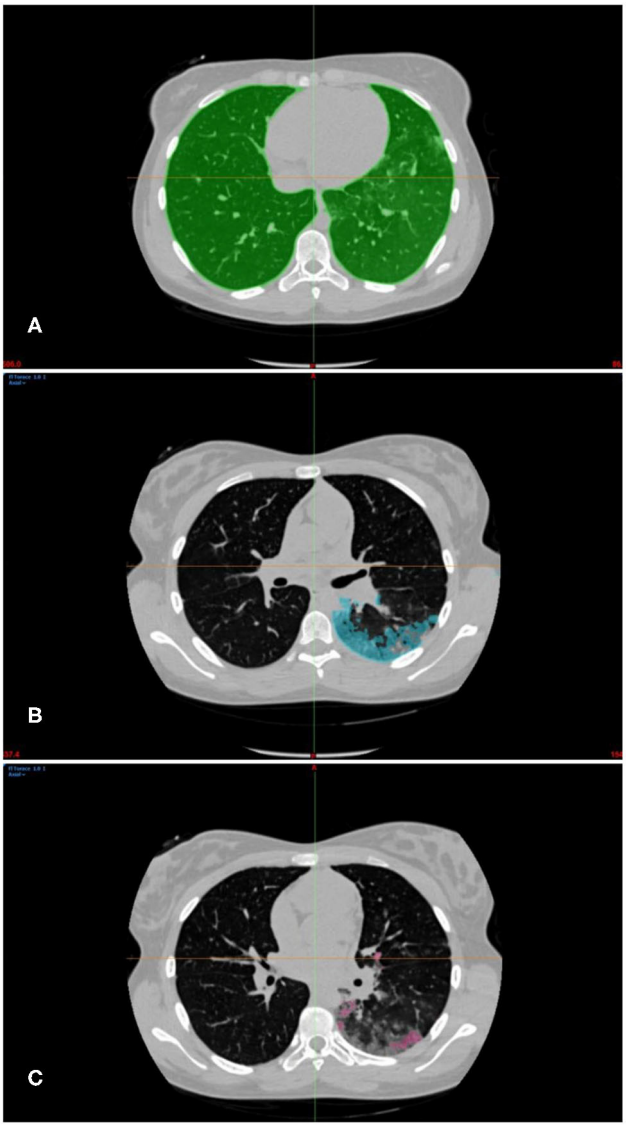


FIGURE 1 | (A) Green mask of normal parenchyma segmentation within Hounsfield range −1024Hu to −543Hu. **(B)** Light blue mask of sub-pleural GGO areas segmentation within Hounsfield range between −957Hu to +1246Hu. **(C)** Pink mask spot consolidations segmentation within Hounsfield range between −834Hu to +356Hu.

In order to obtain an adequate 3D rendering and 3D mesh reconstruction, the model was optimized with the Rhinoceros software (McNeel, USA) to allow adequate 3D visualization for clinical purposes.

RESULTS

The 3D reconstructions allowed us to accurately calculate volumes of Lungs, GGO areas, and consolidation spots. A volume-relationship was then established between the different reconstructed parts, as shown in **Table 1**.

TABLE 1 | Quantification of lung volumes obtained from CT 3D reconstructions.

	Total lung	Left lung	Ground glass opacity	Consolidations spots
Volume (ml)	4.724	2.136	240.94	26.30
Total lung Vol (%)	–	45.21	5.10	0.55
Left lung Vol (%)	–	–	11.28	1.23

Ground glass opacity areas were 5.1% of the entire lung volumes and 11.28% of the left lung. Consolidation spots represented 0.55% of the entire lung volumes and 1.23% of the left lung.

3D rendering proved to be a very simple and effective approach for representing the lung anatomy including the distribution/topography of the different pathological areas. We obtained a series of different 3D volume rendering views to highlight radiological changes as shown in **Figures 2A,B**. We also produced a 3D movie (additional Video online) which shows a more comprehensive rotational rendering overview of the parenchymal changes, interstitial pneumonia related. The final rendering was uploaded on a 3D online viewer platform (Sketchfab), which allows the clinical team to explore and navigate the 3D model on a web-link, taking advantage of a very powerful rendering capability with no need for dedicated workstation and additional software.

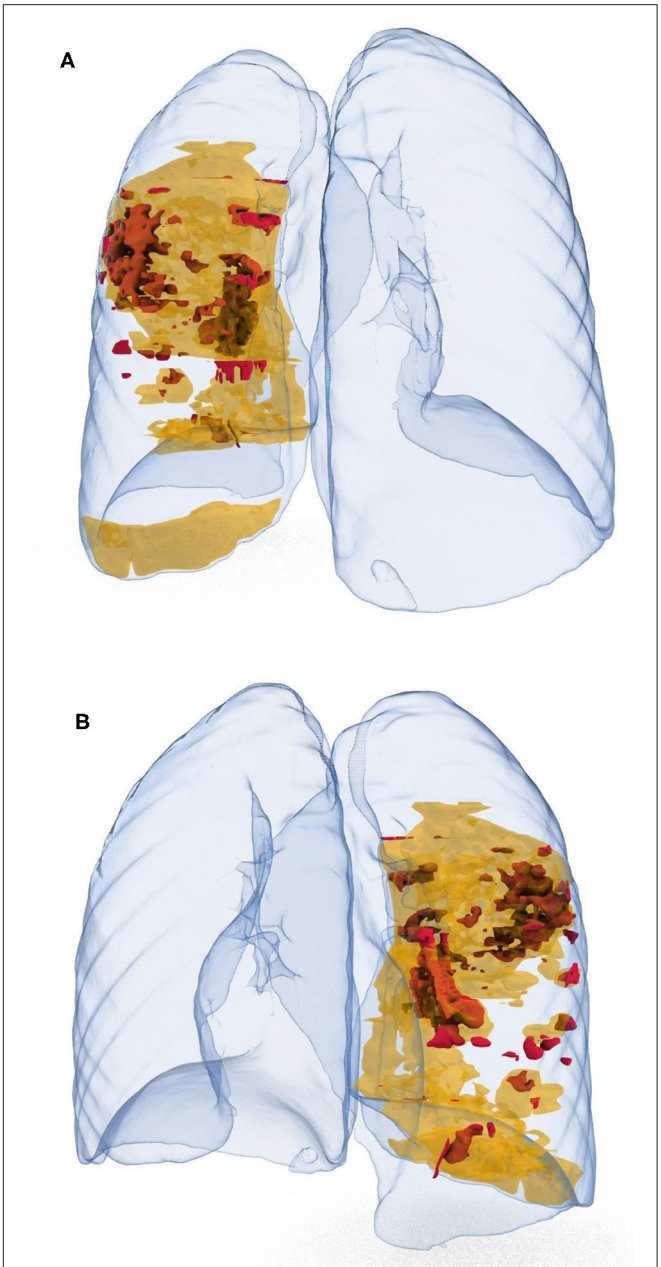


FIGURE 2 | Back view **(A)**, and frontal view **(B)** of lungs parenchyma (crystal clear), with GGO areas in yellow, and spot consolidation in red.

DISCUSSION

3D modeling represents a "paradigm shift" from the classical descriptions of lung changes based on conventional radiological reformats to 3D volume rendering reconstructions, that could improve the understanding (and communication to families and patients) of the extent and nature of these lung changes. In addition, we try to test it as a potential tool for surveillance and comparisons in clinical practice and research activity.

In this case we apply advanced 3D segmentation tools to increase the diagnostic information in case of Covid-19 patient. In effect, the progressive impairment of lung tissues due to the Sars-Cov2 infection is one of the most threatening phenomenon for people affected by this viral infection (1). CT has been introduced in many diagnostic algorhytm to manage these patients in order to predict or anticipate severe clinical deterioration (9, 10). Detailed extent and quantification of affected lung volume, such as GGO or consolidation areas, could be helpful in the management of this pandemic infection. Therefore, accurate quantitative assessment might be useful and included in the CT scan report.

The 3D reconstruction and rendering views in addition to provide a comprehensive and self explaining lung "tissue characterization" with distribution of the different pathological areas, seems to be able to allow reliable lung volumes calculation with detailed analysis of the compromised areas.

We described our algorithm to obtain 3D rendering views and accurate lung volume calculations; it appears to be easily obtainable using a commercial 3D software and potentially reproducible even by using different 3D platforms. In addition, 3D visualization of lung lesions can provide to the medical staff a wider and self-explaining understanding of the underlying lung pathology of Covid-19 patients. As already reported (11) 3D models can also be used for communication with families and patients and for teacing purposes.

Moreover, since CT represents an important imaging biomarker and is pivotal to guide pharmacological management and improve ventilation strategies, further

implementation of semi-automatic and/or fully automatic (AI-based) algorithms for image processing (12, 13) might be beneficial in order to rapidly and systematically provide accurate data about the extent of lung disease in these patients.

Main limitation of our study is that it is a single case investigation. Therefore, we can't assess its reproducibility, and its real impact on clinical practice. Our aim is to point out the opportunity and feasibility of this approach in the particular clinical setting of Covid-19 pandemic in order to stimulate its wider use. Further analysis aimed to assess the clinical impact for the management and risk stratification of this novel tool, and its reproducibility in this setting are obviously needed.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

Written informed consent was obtained from the legal guardian/next of kin of the participant for the publication of this case report and any identifying images or information.

AUTHOR CONTRIBUTIONS

LB and AS: idea for the article. AM: CT-exam. LB: images and video production. AM, AC, FC, MC, and PT: revision and approval of the manuscript. LB, AS, and PC: writing the article. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fped.2020.00453/full#supplementary-material

REFERENCES

1. Zu ZY, Di Jiang M, Xu PP, Chen W, Ni QQ, Lu GM, et al. Coronavirus disease 2019 (COVID-19): a perspective from China. *Radiology*. (2020) 296:200490. doi: 10.1148/radiol.2020200490
2. Maher MM, Kalra MK, Sahani DV, Perumpillichira JJ, Rizzo S, Saini S, et al. Techniques, clinical applications and limitations of 3D reconstruction in CT of the abdomen. *Korean J Radiol.* (2004) 5:55–67. doi: 10.3348/kjr.2004.5.1.55
3. Le Moal J, Peillon C, Dacher JN, Baste JM. Three-dimensional computed tomography reconstruction for operative planning in robotic segmentectomy: a pilot study. *J Thorac Dis.* (2018) 10:196–201. doi: 10.21037/jtd.2017.11.144
4. Sánchez-Sánchez Á, Girón-Vallejo Ó, Ruiz-Pruneda R, Fernandez-Ibieta M, García-Calderon D, Villamil V, et al. Three-Dimensional printed model and virtual reconstruction: an extra tool for pediatric solid tumors surgery. *Eur J Pediatr Surg Reports*. (2018) 6:e70–6. doi: 10.1055/s-0038-1672165
5. Biglino G, Capelli C, Bruse J, Bosi GM, Taylor AM, Schievano S. Computational modelling for congenital heart disease: how far are we from clinical translation? *Heart*. (2017) 103:98–103. doi: 10.1136/heartjnl-2016-310423
6. Xia W, Shao J, Guo Y, Peng X, Li Z, Hu D. Clinical and CT features in pediatric patients with COVID-19 infection: different points from adults. *Pediatr Pulmonol.* (2020) 55:1169–74. doi: 10.1002/ppul.24718
7. Mansoor A, Bagci U, Foster B, Xu Z, Papadakis GZ, Folio LR, et al. Segmentation and image analysis of abnormal lungs at CT: current approaches, challenges, and future trends. *Radiographics*. (2015) 35:1056–76. doi: 10.1148/rg.2015140232
8. Stein JM, Walkup LL, Brody AS, Fleck RJ, Woods JC. Quantitative CT characterization of pediatric lung development using routine clinical imaging. *Pediatr Radiol.* (2016) 46:1804–12. doi: 10.1007/s00247-016-3686-8
9. Xu X, Yu C, Qu J, Zhang L, Jiang S, Huang D, et al. Imaging and clinical features of patients with 2019 novel coronavirus SARS-CoV-2. *Eur J Nucl Med Mol Imaging.* (2020) 47:1275–80. doi: 10.1007/s00259-020-04735-9
10. Bernheim A, Mei X, Huang M, Yang Y, Fayad ZA, Zhang N, et al. Chest CT Findings in coronavirus disease-19 (COVID-19): relationship to duration of infection. *Radiology*. (2020) 295:200463. doi: 10.1148/radiol.2020200463
11. Biglino G, Capelli C, Wray J, Schievano S, Leaver L-K, Khambadkone S, et al. 3D-manufactured patient-specific models of congenital heart defects for communication in clinical practice: feasibility and acceptability. *BMJ Open*. (2015) 5:e007165. doi: 10.1136/bmjopen-2014-007165
12. Xu M, Qi S, Yue Y, Teng Y, Xu L, Yao Y, et al. Segmentation of lung parenchyma in CT images using CNN trained with the clustering algorithm generated dataset 08 information and computing sciences 0801 artificial intelligence and image processing robert koprowski. *Biomed Eng Online.* (2019) 18:1–21. doi: 10.1186/s12938-018-0619-9
13. Saba T. Automated lung nodule detection and classification based on multiple classifiers voting. *Microsc Res Tech.* (2019) 82:1601–9. doi: 10.1002/jemt.23326

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer KM declared a past co-authorship with several of the authors AS and TS to the handling editor.

frontiers in Pediatrics

BRIEF RESEARCH REPORT
published: 06 August 2020
doi: 10.3389/fped.2020.00517

Child Healthcare and Immunizations in Sub-Saharan Africa During the COVID-19 Pandemic

Danilo Buonsenso[1,2]*, *Bianca Cinicola*[3], *Memenatu Ngaima Kallon*[4] and *Francesco Iodice*[5,6]

[1] Department of Woman and Child Health and Public Health, Fondazione Policlinico Universitario A. Gemelli IRCCS, Rome, Italy, [2] Istituto di Microbiologia, Università Cattolica del Sacro Cuore, Rome, Italy, [3] Department of Pediatrics, Sapienza University of Rome, Policlinico Umberto I, Rome, Italy, [4] Ministry of Health and Sanitation, Freetown, Sierra Leone, [5] Institute of Neurology, Fondazione Policlinico Universitario A. Gemelli IRCCS, Rome, Italy, [6] Department of Neuroscience and Neurorehabilitation, San Raffaele Pisana IRCCS, Rome, Italy

Since COVID-19 in the pediatric population is infrequently severe, the indirect costs of the pandemic, related to the measures implemented to deal with the spread of the virus, can be worse than the infection itself. To assess this issue, we evaluated the number of children vaccinated or evaluated for the most common diseases in a poor village in Sierra Leone, showing a worrisome drop in vaccinations performed and children evaluated for acute diseases. Our preliminary findings highlight that support is needed to guarantee basic services to children during the COVID-19 pandemic, particularly in poor settings where preventive measures can be lifesaving in the long term.

Keywords: COVID-19, Africa, SARS-CoV-2, vaccination, children, malaria, pneumonia

OPEN ACCESS

Edited by:
Valeriane Leroy, Institut National de la Santé et de la Recherche Médicale (INSERM), France

Reviewed by:
Ulrich von Both, Ludwig Maximilian University of Munich, Germany
Sabah Boufkhed, King's College London, United Kingdom

***Correspondence:**
Danilo Buonsenso
danilobuonsenso@gmail.com

Specialty section:
This article was submitted to Pediatric Infectious Diseases, a section of the journal Frontiers in Pediatrics

Received: *12 May 2020*
Accepted: *22 July 2020*
Published: *06 August 2020*

Citation:
Buonsenso D, Cinicola B, Kallon MN and Iodice F (2020) Child Healthcare and Immunizations in Sub-Saharan Africa During the COVID-19 Pandemic. Front. Pediatr. 8:517. doi: 10.3389/fped.2020.00517

INTRODUCTION

After the first description of clusters of pneumonia due to SARS-CoV-2 in China, the virus has spread all over the world. Monitoring activities and quarantine are playing an important role as rapid corrective strategies, but these processes could be very critical in the world's poorest health systems. Since COVID-19 in the pediatric population is infrequently severe (1), the indirect costs of the pandemic, related to the measures implemented to deal with the spread of the virus, can be worse than the infection itself.

In this scenario, the COVID-19-related effects on child healthcare in Africa can be massive (2), especially in the remote areas of sub-Saharan Africa, where health, economic, and social monitoring systems are poorly developed. This situation may even worsen, considering that several countries are soon entering the rainy season, which has major consequences for daily life and a potential impact on the rate of SARS-CoV-2 spread. A decrease in vaccinations could be among the most feared indirect effects of the pandemic, leading to a possible increase in morbidity and mortality in a population that has been considered to be less involved as regards the severe clinical manifestations of SARS-CoV-2 compared to adults (1).

For these reasons, we aimed to understand the potential indirect impact of COVID-19 on child vaccinations and basic healthcare in a typical poor peripheral area of Sierra Leone (Kent, Rural Western Area, Sierra Leone). This area is not provided with basic services such as electricity and running water, with people needing to go to local streams for daily provision of water (3, 4). Moreover, the area, although not far from the capital, Freetown, refers to local health centers for vaccinations as well as for routine antenatal and postnatal care. The area has an overall 90% vaccination coverage, which is in line with the overall national data published by the World

TABLE 1 | Vaccination performed in a community health center in Sierra Leone during COVID-19 lockdown, compared with the previous year.

Vaccination	Children under 5 years of age			
	01/03/2019 to 26/04/2019	01/03/2020 to 26/04/2020	Change (%)	*P*-value
BCG	36	17	−52.7	$p < 0.0005$
OPV0	36	17	−52.7	$p < 0.0005$
OPV1	58	17	−70.7	$p < 0.0005$
PENTA1	58	17	−70.7	$p < 0.0005$
PCV1	58	17	−70.7	$p < 0.0005$
ROTA1	58	17	−70.7	$p < 0.0005$
OPV2	71	15	−78.9	$p < 0.0005$
PENTA2	71	15	−78.9	$p < 0.0005$
PCV2	71	15	−78.9	$p < 0.0005$
ROTA2	71	15	−78.9	$p < 0.0005$
IPTI1	49	15	−69.4	$p < 0.0005$
OPV3	67	15	−77.6	$p < 0.0005$
PENTA3	67	15	−77.6	$p < 0.0005$
PCV3	67	15	−77.6	$p < 0.0005$
IPTI2	44	15	−65.9	$p < 0.0005$
IPV	67	15	−77.6	$p < 0.0005$
IPTI3	45	22	−51.1	$p < 0.0005$
Measles	64	22	−65.6	$p < 0.0005$
Yellow fever	64	22	−65.6	$p < 0.0005$
Measles 2nd	49	8	−83.7	$p < 0.0005$

BCG, Bacille Calmette Guerin; OPV, oral polio vaccine; PENTA, diphtheria, pertussis, tetanus, hepatitis B, and hemophilus; PCV, pneumococcal vaccine; ROTA, rotavirus; TT, tetanus toxoid vaccine; IPTi, intermittent preventive treatment in infants. The number associated with the abbreviation denotes initial and booster vaccinations. Data are collected as absolute numbers.

TABLE 2 | Main acute illnesses evaluated in a community health center in Sierra Leone during COVID-19 lockdown compared with the previous year.

Diseases	Children under 5 years of age			
	01/03/2019 to 26/04/2019	01/03/2020 to 26/04/2020	Change (%)	*P*-value
Malaria (clinical diagnosis)	211	126	−40.3	$P > 0.05$
Malaria (confirmed)	120	90	−25	$P > 0.05$
Pneumonia	129	74	−42.6	$P > 0.05$
Diarrhea	30	15	−50	$P > 0.05$
Death	0	0	/	na

Data were collected as absolute numbers.

Health Organization and UNICEF in 2019 (http://158.232.12.119/immunization/monitoring_surveillance/data/sle.pdf). This research is part of a project that we started in 2015 in Bureh Town, Rural Western Area, Sierra Leone, partnering with the nearby community health center of Kent (Rural Western Area, Sierra Leone), which is the referral center for basic pediatric health services, from child visits (health controls and acute diseases) to vaccinations.

In Sierra Leone, the first restrictive governmental measures (border control and closure, social distancing) were dated March 23, with total lockdown declared on April 3 (https://www.imf.org/en/Topics/imf-and-covid19/Policy-Responses-to-COVID-19#S). As in many African countries, healthcare in Sierra Leone is free only for children under 5 years and pregnant women. Moreover, since health centers cover a wide area, most people need to pay for transport services to reach health centers, which is generally difficult and is even harder after COVID-19-related drop in income. Also, quarantine and fear of contagion, as well as the fear of being recognized as a COVID-19 case and stigmatized [a known problem in Africa, as already described with HIV and Tuberculosis (4)] might impact the routine child healthcare in these settings.

METHODS

We performed a retrospective cross-sectional study collecting the number of children under 5 years of age vaccinated for the most common diseases at the Kent Community Health Post (referral from the local communities of Kent, Bureh, Checkpoint, Bonga Wharf, and Quarry; estimated population of about 5,000 people) from March 1, 2020, to April 26, 2020, and compared the results to the same period of the previous year (March 1, 2019, to April 26, 2019). In the same periods, the diagnoses of malaria, pneumonia, and diarrhea were also assessed for the two different populations of children under 5 years of age as well as immunization for tetanus in pregnant women. During the lockdown, the health center continued to provide the same activities as in the pre-lockdown era, with all services guaranteed, from acute disorders to immunization services. Therefore, there was no reduction in the health activities offered to the local population due to COVID-19 restrictions. The data were collected, as absolute numbers, by the center's health workers in accordance with the usual rules held in Sierra Leone. Cases were retrospectively obtained using the health facility routine activity reporting forms. In many peripheral Sub-Saharan Africa health centers, data are collected by the health facility on a register by crossing off with a pen the type of vaccinations performed or the disease diagnosed, without registering personal data. Peripheral health centers have a registry book with dedicated pages for registration of diagnoses and type of vaccinations. For example, they have boxes to tick labeled "PENTA1," "PENTA2," and "PENTA3." This means that "PENTA1" is the first, and the others are the boosters.

Written data were subsequently transferred to an electronic database and analyzed with SPSS software v.36. A comparison between the number of vaccinated children in the two periods of time, for each type of vaccine, was performed by applying a Wilcoxon test.

The study was approved by a local commission composed of the research team of the Kent Community Health Post, the headman of the community, and the old men of the village, in a similar way as happens for all important political and economic

decisions in the examined area (n24_may-2020). Personal data were not collected.

RESULTS

We noticed that a lower number of children received vaccination in 2020 compared with 2019, ranging from 50 to 85% depending on the individual vaccine analyzed, including BCG and OPV1, which are given directly at birth in Sierra Leone (see **Table 1** for full details). Moreover, we also noticed a drop in common diagnoses, as has been happening also in developed countries (5), and a reduction of the most common clinical conditions (malaria, pneumonia, and diarrhea), although no increases in deaths were reported (**Table 2**). There was a 50–80% drop in vaccination in 2020 compared to the previous year ($p < 0.0005$). Conversely, although the number of common diagnoses was lower in 2020, there was not enough evidence to be sure that this is a true difference ($p > 0.05$).

DISCUSSION

Although we only analyzed preliminary data from an extremely poor area, they show an important decline in the vaccination rate and, although the difference was not significant, in child visits for acute diseases in the lockdown period in a rural area of Sierra Leone. The reduction in vaccination rates is particularly evident for booster vaccinations, suggesting that parents are not bringing their children back to health facilities for the subsequent controls and vaccinations, possibly because of fear of contagion. It is important to remark also the reduction of vaccinations given at birth in Sierra Leone in our sample (BCG and OPV1). This suggests either a decrease in the number of births or an increase in deliveries at home due to a fear of being infected in health facilities, which may be a high risk for woman and child health. These data highlight the need for active surveillance of births and immunizations registered in peripheral health centers in order to better understand the meaning of our findings. No increase in deaths in the area was reported. Anecdotical reports from our team highlighted no problems regarding vaccine supplies in the area due to COVID-19 restrictions, so the reduced vaccinations are not related to vaccine supplies. Also, it is important to highlight that, during the lockdown, the health center maintained the same activities as in the pre-lockdown era, with all services guaranteed, from acute disorders to immunizations services. Therefore, there was no reduction of the health activities offered to the local population because of COVID-19 restrictions.

We are aware of the limitations of our findings. In particular, this is a retrospective study concerning a limited time period, we collected absolute numbers, and no comprehensive epidemiological data for the area are currently available (such as birth rates from local villages) since the current pandemic is creating a high workload for local workers and, at the same time, limits their possibility of interacting with other offices. However, we also understand that collecting more robust data in very remote, peripheral, rural areas of Sierra Leone is not easy, particularly in a period when movements between areas are not allowed. However, these findings are new and bear potential significant implications. Local health workers anecdotally reported they had the feeling that people could not afford routine care (from transport fees to visit fees for those over 5 years of age), and our data provide, to the best of our knowledge, the first evidence of a possible drop in access for basic but priority pediatric services in remote areas, while similar rates have already been reported in the adult population of South Africa (6). A similar scenario is currently happening in the United States, where a recent nationwide analysis of vaccine information found that measles, mumps, and rubella vaccinations had dropped by 50% during the COVID-19 outbreak (6). However, while the U.S. has tools to face this challenge, and the American Academy of Pediatrics is working hard to support pediatricians and families to ensure vaccination coverage, low-income rural/peripheral settings in Sierra Leone do not have easy access to the appropriate instruments to ensure that families bring children to health centers, and our data on a small sample population confirm what the World Health Organization predicted as one of the possible effects of the lockdown on child healthcare (7, 8). Importantly, a drop in vaccination coverage in Africa bears potentially devastating consequences for child health.

Although our data must be interpreted with caution, since we report only a single experience from an area of Sierra Leone, they clearly indicate a new scenario. It is not easy for low-income rural/peripheral settings in Sierra Leone to respond to COVID-19, giving rise to a potential risk that children could suffer from the indirect consequences of the pandemic more than in other parts of the world. Sources of income in peripheral/rural areas in Sierra Leone can be significantly impacted by the COVID-19 pandemic lockdown (9). Although testing, isolating, and quarantining are the best ways to prevent COVID-19 diffusion in Africa (10), this can lead to difficulties in providing basic services to the local population, as previously described (3). People from the area we analyzed are already experiencing limitations related to the pandemic in accessing basic needs, such as food, water, health services, and preventive strategies, and are now depending on donations for basic food (3). Highly vulnerable populations, such as younger children, often already compromised by malnutrition and comorbidities, face a greater risk of developing diseases or missing preventive opportunities (11). In these peripheral/rural areas in Sierra Leone, people need a comprehensive approach that aims to provide COVID-19 care facilities as well as social services and essential resources for more common problems, and economic support must not be directed only to epidemiologic research trials (12). Importantly, the rainy season is coming soon, and the situation will probably worsen.

In conclusion, despite the limitations mentioned, our findings highlight a possible drop in immunizations in children living in a peripheral, rural area in Sierra Leone, West Africa. Considering the potential short- and long-term impact of reduced immunization rates on child health, there is a need to actively monitor vaccination practices in low-resource settings to ensure that there is no break in service delivery.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The study was approved by a local commission composed of the research team of the Kent Community Health Post, the headman of the community, and the old men of the village, in a similar way as happens for all important political and economic decisions in the examined area (n24_may-2020). Personal data were not collected.

AUTHOR CONTRIBUTIONS

DB, BC, and FI substantial contributions to the conception or design of the work. DB and MK were responsible for acquisition, analysis, or interpretation of data for the work. DB, BC, and FI drafted the work or revising it carefully for important intellectual content. All author gave final approval of the version to be published and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

ACKNOWLEDGMENTS

We are grateful to all of our colleagues who supported the development of local health services and training of community health workers in the Western Rural Area of Sierra Leone and, in particular, Ismail Jaber, Matilda Yamba, Prince Williams, MK, Nee Turay, Pietro Sollena, Francesca Vassallo, Vittorio Sabatino, Mara Caramia, Gregory Leeb, Valeria Pansini, Filippo Bruno, Andrea Deidda, Arianna Cafarotti, Mattia Belardinelli, Alessia De Nisco, Stefano Rocchi, Irene Sollena, Davide Guglielmi, Maria Giulia Conti, Jessica Balerna, Francesco Madeddu, Daniele Barbuto, Luigi Torricelli, and Rachel Mannings. We are also grateful to Cassa Galeno, which granted DB a project aimed at the development of the Italian-Sierra Leone research network in pediatrics.

REFERENCES

1. Parri N, Lenge M, Buonsenso D, Coronavirus Infection in Pediatric Emergency Departments (CONFIDENCE) Research Group. Children with Covid-19 in pediatric emergency departments in Italy. *N Engl J Med*. (2020) 383:187–90. doi: 10.1056/NEJMc2007617
2. El-Sadr WM, Justman J. Africa in the path of Covid-19. *N Engl J Med*. (2020) 383:e11. doi: 10.1056/NEJMp2008193
3. Buonsnso D, Cinicola B, Raffaelli F, Sollena P, Iodice F. Social consequences of COVID-19 in a low resource setting in Sierra Leone, West Africa. *Int J Infect Dis*. (2020) 97:23–6. doi: 10.1016/j.ijid.2020.05.104
4. Buonsenso D, Iodice F, Sorba Biala J, Goletti D. COVID-19 effects on tuberculosis care in Sierra Leone. *Pulmonology*. (2020). doi: 10.1016/j.pulmoe.2020.05.013. [Epub ahead of print].
5. Bressan S, Buonsenso D, Farrugia R, Parri N, Oostenbrink R, Titomanlio L, et al. Preparedness and response to Pediatric CoVID-19 in European Emergency Departments: a survey of the REPEM and PERUKI networks. *Ann Emerg Med*. (2020). doi: 10.1016/j.annemergmed.2020.05.018. [Epub ahead of print].
6. Siedner MJ, Kraemer JD, Meyer MJ, Harling G, Mngomezulu T, Gabela P, et al. Access to primary healthcare during lockdown measures for COVID-19 in rural South Africa: a longitudinal cohort study. *medRxiv*. (2020). doi: 10.1101/2020.05.15.20103226. [Epub ahead of print].
7. Hoffman J. Vaccine rates drop dangerously as parents avoid doctor's visits. *The New York Times*. (2020). Available online at: https://www.nytimes.com/2020/04/23/health/coronavirus-measles-vaccines.html (accessed May 3, 2020).
8. *At Least 80 Million Children Under One at Risk of Diseases Such as Diphtheria, Measles and Polio as COVID-19 Disrupts Routine Vaccination Efforts*. Warn Gavi, WHO, UNICEF. Available online at: https://www.who.int/news-room/detail/22-05-2020-at-least-80-million-children-under-one-at-risk-of-diseases-such-as-diphtheria-measles-and-polio-as-covid-19-disrupts-routine-vaccination-efforts-warn-gavi-who-and-unicef (accessed July 2, 2020).
9. Renzaho AMN. The need for the right socio-economic and cultural fit in the COVID-19 Response in Sub-Saharan Africa: examining demographic, economic political, health, and socio-cultural differentials in COVID-19 morbidity and mortality. *Int J Environ Res Public Health*. (2020) 17:3445. doi: 10.3390/ijerph17103445
10. Fanidi A, Jouven X, Gaye B. Strategies to control COVID-19 and future pandemics in Africa and around the globe. *Eur Heart J*. (2020). doi: 10.1093/eurheartj/ehaa278. [Epub ahead of print].
11. Samiak L, Emeto TI. Vaccination and nutritional status of children in Karawari, East Sepik Province, Papua New Guinea. *PLoS ONE*. (2017) 12:e0187796. doi: 10.1371/journal.pone.0187796
12. Baker T, Schell CO, Petersen DB, Sawe H, Khalid K, Mndolo S, et al. Essential care of critical illness must not be forgotten in the COVID-19 pandemic. *Lancet*. (2020) 395:1253–4. doi: 10.1016/S0140-6736(20)30793-5

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular and Infection Microbiology

BRIEF RESEARCH REPORT
published: 06 August 2020
doi: 10.3389/fcimb.2020.00445

Value of Viral Nucleic Acid in Sputum and Feces and Specific IgM/IgG in Serum for the Diagnosis of Coronavirus Disease 2019

Yuwen He[1†], Jiangyan Luo[2†], Jie Yang[2†], Jinlong Song[3], Li Wei[1] and Weifeng Ma[2*]*

[1] Department of Pharmacy, The First Affiliated Hospital of Guangzhou Medical University, Guangzhou, China, [2] Department of Microbiology, School of Public Health, Southern Medical University, Guangzhou, China, [3] Department of Clinical Laboratory, The First Affiliated Hospital of Guangzhou Medical University, Guangzhou, China

A new type of coronavirus-induced pneumonia eventually termed "coronavirus disease 2019" (COVID-19) was diagnosed in patients in Wuhan (Hubei Province, China) in December 2019, and soon spread worldwide. To improve the detection rate of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), we analyzed the results of viral nucleic acid and serum-specific antibody tests on clinical samples from 20 patients with SARS-CoV-2 infection diagnosed at the First Affiliated Hospital of Guangzhou Medical University in China. By comparing various sample types collected from COVID-19 patients, we revealed multiple pathways for SARS-CoV-2 shedding, and a prolonged detectable period for viral nucleic acid test in sputum specimens, demonstrating that the timeline of the viral shedding is of great value in determining the time of release from quarantine or discharge from hospital. We also recommend for the application of serological test to assist in confirming SARS-CoV-2 infection judged by viral nucleic acid test, especially when COVID-19-related symptoms have appeared and the viral nucleic acid test was negative. Our findings are critical for the diagnosis of SARS-CoV-2 infection and for determining deadline of restriction measures to prevent transmission caused by convalescent patients with COVID-19.

Keywords: SARS-CoV-2, COVID-19, sputum, feces, nucleic acid test, serological test, IgM, IgG

OPEN ACCESS

Edited by:
Rong Fang,
The University of Texas Medical Branch at Galveston, United States

Reviewed by:
Sabrina John Moyo,
University of Bergen, Norway
Kai Huang,
The University of Texas Medical Branch at Galveston, United States

***Correspondence:**
Li Wei
runkingone@126.com
Weifeng Ma
maweifeng919@163.com

[†] These authors have contributed equally to this work

Specialty section:
This article was submitted to Clinical Microbiology, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: *10 April 2020*
Accepted: *20 July 2020*
Published: *06 August 2020*

Citation:
He Y, Luo J, Yang J, Song J, Wei L and Ma W (2020) Value of Viral Nucleic Acid in Sputum and Feces and Specific IgM/IgG in Serum for the Diagnosis of Coronavirus Disease 2019. Front. Cell. Infect. Microbiol. 10:445. doi: 10.3389/fcimb.2020.00445

INTRODUCTION

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), identified in Wuhan, China at the end of 2019, spread rapidly worldwide. In order to diagnose a large number of patients, samples of lower respiratory tract such as sputum with high positive rate are generally collected for viral nucleic acid detection (Han et al., 2020; Qu et al., 2020). In addition, some studies have reported the presence of viruses in feces (Tang et al., 2020; Wu et al., 2020), implying the risk of fecal-oral transmission, and indicating that specimen collection should not be limited to respiratory samples. Common symptoms at onset of illness were fever (98%), cough (76%), myalgia, or fatigue (44%) (Huang et al., 2020). However, it is worth noting that carriers who are subclinical can also infect people (Rothe et al., 2020). Therefore, it is necessary to deliver viral nucleic acid and serological tests for people with a history of exposure to SARS-CoV-2. We undertook a study on the viral nucleic acids of SARS-CoV-2 in swabs (nasal, pharyngeal), sputum and feces, as well as antibodies in the serum of COVID-19 patients admitted to the First Affiliated Hospital of Guangzhou Medical

University, China. We aimed to clarify the importance of the test results of different specimen types for the diagnosis and de-isolation of patients with coronavirus disease 2019 (COVID-19).

METHODS

Ethical Approval of the Study Protocol

The study protocol was approved by the Medical Ethics Committee of the First Affiliated Hospital of Guangzhou Medical University (Guangzhou, China). Written informed consent was obtained from all study participants.

Collection and Processing of Specimens

From 29 January to 6 June 2020, samples (swabs, feces, sputum, and blood) of 20 COVID-19 patients were collected from the First Affiliated Hospital of Guangzhou Medical University, a designated hospital for the treatment of critically ill patients with COVID-19 in Guangzhou, a first-tier city in China. By the way, there were only 20 patients in this hospital who were almost critically ill or severe, other mild or asymptomatic patients were not admitted. The subjects of our study were all COVID-19 patients admitted to our hospital, and there were no additional inclusion or exclusion criteria. After admission, the patient was tested for viral nucleic acid continuously for a week and then every 2–3 days. Antibodies have been tested every 2–3 days since 15 February. To improve the efficiency of clinical work, collection of long-lasting negative specimens was suspended, while positive specimens were continuously collected until discharge. In the First Affiliated Hospital of Guangzhou Medical University, extraction of nucleic acids from the respiratory and fecal samples was performed with the commercialized nucleic acid extraction kits (Magnetic bead method, Daan Gene Co., Ltd. of Sun Yat-sen University, Guangzhou, China). The operations were completely following the instruction of the kit. The blood specimen was centrifuged with 3,000 rpm for 15 min to separate the serum within 24 h after collection, and then inactivated at 56°C for 30 min and stored at 4°C until use.

RT-PCR

RNA was detected using the New Coronavirus 2019-nCoV Nucleic Acid Detection kit (Daan Gene Co., Ltd. of Sun Yat-sen University, Guangzhou, China). The analytical sensitivity of our kit was 500 copies/mL. This kit had no cross-reaction with other pathogens similar to SARS-CoV-2 or causing similar symptoms. Exogenous substances such as COVID-19 therapeutic drugs and endogenous substances such as blood and mucus in specimens did not interfere with the detection results of the kit. The primer and probe sequences designed for the open reading frame (ORF1ab), nucleoprotein (N) gene regions of SARS-CoV-2 are shown in **Table 1**. NC (ORF1ab/N) PCR reaction solution A and solution B were oscillated thoroughly after melting at room temperature followed by 8,000 rpm centrifugation for several seconds. Next, 17 μL of NC (ORF1ab/N) PCR reaction solution A and 3 μL of NC (ORF1ab/N) PCR reaction solution B were fully mixed and centrifuged briefly to bring down all the liquid to the bottom of the tube. Each PCR reaction tube was added with 20 μL of the above amplification system followed by adding 5 μL of the negative quality control (QC) substance, the viral nucleic acid of the sample to be tested, or positive QC substance. It was a one-step RT-PCR. The total PCR reaction volume was 25 μL. The volume of the template was 5 μL. The concentration of primers and probes was 1 μM and 10 μM in the final PCR reaction volume respectively. These PCR reaction tubes were centrifuged (8,000 rpm) for a few seconds at room temperature and placed in an RT-PCR instrument (ABI Prism 7500; Applied Biosystems, Foster City, CA, USA) for amplification and detection. The amplification process was as follows: 50°C for 15 min, and 95°C for 15 min, then 45 cycles were performed, including 94°C for 15 s, 55°C for 45 s, and the default melting curve steps of the RT-PCR instrument. If the Ct value of the tested sample was < 40 in the FAM and VIC channels (FAM and VIC are fluorescent dyes), and there was an obvious amplification curve, the sample was judged to be positive for SARS-CoV-2.

Serological Test

Our serological test used for SARS-CoV-2 specific IgM/IgG antibodies was a rapid detection method. The detection principle of the IgM/IgG antibody detection kit (Livzon Diagnostics Co., Ltd, Zhuhai, China) for SARS-CoV-2 is based on colloidal gold immunochromatography. The antibody detection kit was proved that there was no cross-reaction to the kit in the detection of IgM/IgG-positive samples of similar or other viruses. It has been experimentally verified that a variety of exogenous and endogenous substances have not interfered with our antibody detection kit, and the presence of the SARS-CoV-2 IgG antibody does not affect the detection of SARS-CoV-2 IgM antibody and vice versa. The clinical test results of this kit showed that the detection sensitivity of IgM was 79.0% and the specificity was 99.7%; the detection sensitivity of IgG was 84.3% and the specificity was 99.4%; the combined detection sensitivity of IgM and IgG was 90.6% and the specificity was 99.2%. Serum (10 μL) was added to the sample well of the IgM and IgG detection cards. Then, two drops (∼100 μL) of sample diluent were added vertically. If the detection line and QC line appeared within 15 min, then the sample was judged to be positive.

Statistical Analyses

Data processing was carried out using SPSS v22.0 (IBM, Armonk, NY, USA). The differences between samples or individuals were analyzed by ANOVA of randomized block design data and Bonferroni multiple comparison. Data are presented as mean ± SD. Time from onset to admission and hospital stay are presented as median (IQR). ANOVA of randomized block design data with 2-sided $P < 0.05$ was considered significant. Multiple comparison with 2-sided $P < 0.0083$ was considered significant.

RESULTS

Characteristics of Cases

A total of 22 patients were diagnosed as COVID-19 in our hospital, of which two (patients #19, #20) were transferred the next day after hospital admission. The median time from onset to admission to our hospital for 20 patients was 9.5 (7.5–14.0), some patients had been treated elsewhere during this period.

TABLE 1 | Primer and probe sequences.

Genes	Primer sequences		Probe sequences
	Forward	Reverse	
ORF1ab	CCCTGTGGGTTTTACACTTAA	ACGATTGTGCATCAGCTGA	5′-FAM-CCGTCTGCGGTATGTGGAAAGGTTATGG-BHQ1-3′
N	GGGGAACTTCTCCTGCTAGAAT	CAGACATTTTGCTCTCAAGCTG	5′-FAM-TTGCTGCTGCTTGACAGATT-TAMRA-3′

And the median hospital stay was 63.5 (28.0–93.8) days. In fact, a longer time from onset to admission may result in more serious symptoms or organic damages, thereby increasing the difficulty of clinical cure (Qi et al., 2020). Of 20 patients with COVID-19, the mean age were 57.35 years old. The ratio of males: females was 14:6. According to the clinical diagnosis, the ratio of patients with critical: severe: mild disease was 15:2:3. The clinical characteristics were summarized in **Table 2**. During hospitalization, the most common symptoms of 20 patients were fever (20 [100%]), cough (17 [85%]), shortness of breath (15 [75%]), sputum production (13 [65%]), and fatigue (10 [50%]); less common symptoms were headache (3 [15%]) and diarrhea (2 [10%]). In addition, 17 (85%) patients had comorbidities, including 14 (70%) acute respiratory distress syndrome, 9 (45%) myocardial damage, and 8 (40%) hypertension, etc. Specimens were collected from these 20 patients admitted to the hospital since January 29. As of May 10, all 20 patients had turned negative for viral nucleic acid. The discharge criteria include: (i) Body temperature returned to normal for more than 3 days; (ii) Respiratory symptoms were significantly improved; (iii) Chest images showed that acute exudative lesions were significantly improved; (iv) Viral nucleic acid tests were negative in sputum, nasopharyngeal swabs, and feces samples for two consecutive times (at intervals of more than 24 h). Seventeen patients had been discharged from the hospital on June 7, while three patients (patients #8, #13, #22) had not been discharged so far because of other underlying diseases. Among the 20 tested patients, the time-dependent diagnostic results of SARS-CoV-2 RNA in throat swabs, nasal swabs, sputum, and feces were summarized in **Figure 1**, as well as serum specific IgM/IgG antibodies. When all types of specimens turned negative, the symptoms of most patients had completely disappeared or were alleviated.

TABLE 2 | Clinical characteristics of patients infected with COVID-19.

	Patients ($n = 20$)
Signs and symptoms during hospitalization	
Fever	20 (100%)
Cough	17 (85%)
Shortness of breath	15 (75%)
Sputum production	13 (65%)
Fatigue	10 (50%)
Headache	3 (15%)
Diarrhea	2 (10%)
Comorbidities	
Acute respiratory distress syndrome	14 (70%)
Myocardial damage	9 (45%)
Hypertension	8 (40%)
Respiratory failure	8 (40%)
Sepsis	6 (30%)
Diabetes	6 (30%)
Kidney disease	5 (25%)
Dysfunction or abnormal blood coagulation	4 (20%)
Shock	4 (20%)
Chronic liver disease	4 (20%)
Chronic lung disease	3 (15%)

Data are n (%).

High Detection Rate and a Long Positive Duration of SARS-CoV-2 in Sputum Samples

Sputum samples were positive in 19 of the 20 (95%) patients with COVID-19. In other words, of all the sample types tested, the detection rate of the viral nucleic acids of SARS-CoV-2 was highest in sputum samples. Interestingly, SARS-CoV-2 nucleic acid tests in nasal swabs, throat swabs and feces for a small number of patients (patients #10, #14, #17, and #21) were invariably negative from diagnosis to discharge but lasted for 94, 29, 41, and 4 days in sputum, respectively. Moreover, sputum samples remained positive for an average of 42.8 ± 4.2 (mean ± SD) days since diagnosis. The comparison of the virus-carrying duration showed that the persistence of SARS-CoV-2 RNA in sputum stayed significantly longer than that in throat swabs, nasal swabs, and feces (**Figure 2**), which prolonged by 32.0, 24.0, and 20.6 days, respectively. Among them, patient #5 even continued to be positive for 99 days. In short, sputum has a high detection rate of viral nucleic acid and a long-term continuous positive.

Prolonged Presence of SARS-CoV-2 in a Part of Fecal Samples

The feces samples remained SARS-CoV-2 RNA positive for 22.3 ± 29.8 (mean ± SD) days since diagnosis, of which 11 patients (55%) were positive. Although the existence time of virus in feces was not significantly different from that in nasal or throat swabs (**Figure 2**), it is worth noting that patients #2 and #8 presented consecutively positive in feces samples for 24.0 and 29.0 days, respectively after the nasal-swab, throat-swab, and sputum samples consistently became negative. Moreover, patients #5 with the longest positive duration in the sputum had a longer feces duration, which took 103 days before it turned negative.

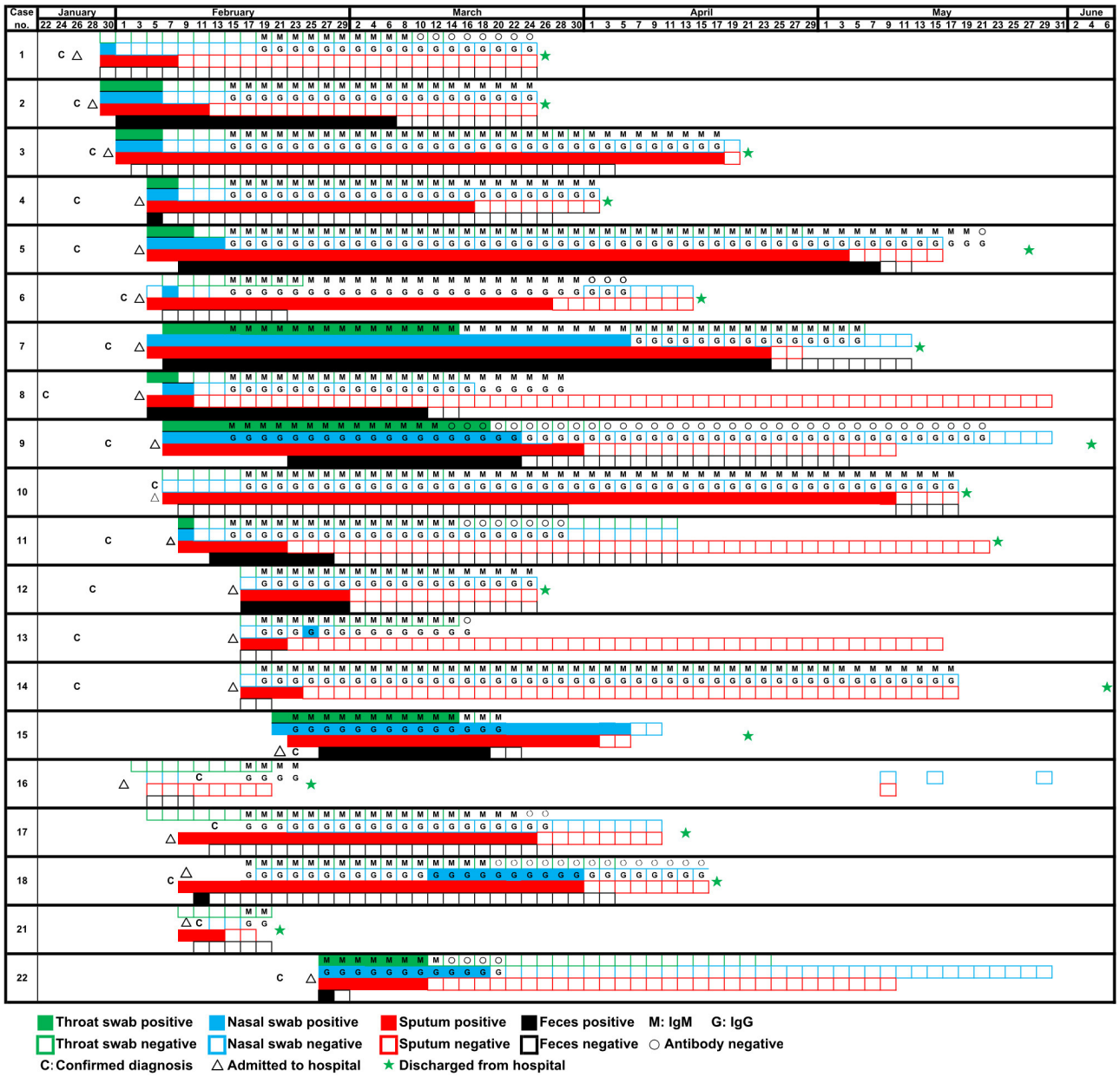


FIGURE 1 | Detection of SARS-CoV-2 RNA of four types of samples (throat swab, nasal swab, sputum, and feces) and specific antibody of 20 patients on different dates.

High Sensitivity of Serological Detection

Although the detection of virus nucleic acid based on RT-PCR has high sensitivity, it is inevitable to produce false-negative results according to our experience and other references (Li et al., 2020). The potential problem of a high false-negative rate caused by the nucleic-acid test prompted us to increase the detection of IgM and IgG antibodies against SARS-CoV-2 from 15 February 2020. These specific antibodies against SARS-CoV-2 were successfully detected in all of 20 patients (100%) (**Figure 1**), with the exception of two patients (patients #19, #20) who were not tested. Otherwise, a few cases such as patient #16 would likely be considered to be SARS-CoV-2-negative through routine RNA testing and, thus, pose a threat to other people. Initially, we observed that patient #16 presented fever and diarrhea, and chest X-ray/CT showed unilateral pulmonary infection. Epidemiological investigation showed a history of passing through the epidemic area and similar symptoms appear on her spouse. For this patient, SARS-CoV-2 RNAs in all kinds of samples were negative while SARS-CoV-2 IgG and IgM remained positive. In view of the above circumstances, we finally took IgM/IgG positive as the diagnostic criteria to include this patient. In a word, we found that the combined detection of viral nucleic

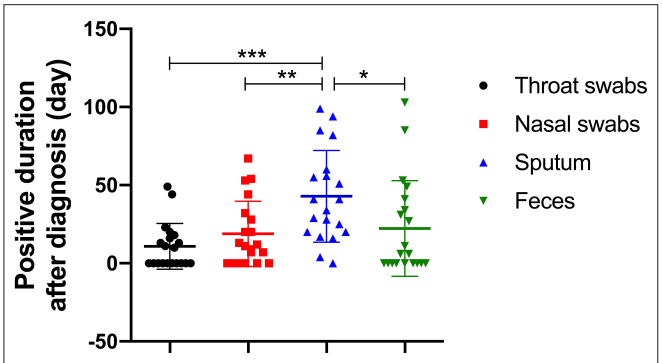


FIGURE 2 | The number of days it took for SARS-CoV-2 RNA to shed in four types of sample (throat swab, nasal swab, sputum, and feces). *$p < 0.00833$, **$p < 0.00167$, ***$p < 0.00017$.

acid and specific antibody successfully improved the detection rate of SARS-CoV-2.

DISCUSSION

In this study, we examined the clinical biological samples of all COVID-19 patients in the first affiliated Hospital of Guangzhou Medical University, most of them were critical or severe patients. Compared with mild patients (Wölfel et al., 2020), our specimens usually have a longer time for virus shedding, and critically ill patients have a longer period of treatment and observation, which provides a reference for the discharge and isolation time of critically ill patients. Secondly, since COVID-19 was first prevalent in China, we successfully demonstrated the whole process from diagnosis to discharge, while other areas may have not detected until the endpoints of some critically ill patients from the beginning of the epidemic to the present. One limitation is that the sample size is relatively small, and more data on critically ill patients need to be collected in the future to further confirm our results. We also call for a follow-up of discharged patients to observe the prognosis or re-positive conditions, although we failed to collect these useful data in time.

Our results show that viral nucleic acid has a high detection rate and a significantly long existence time in the sputum from COVID-19 patients. Research by Qu et al. (2020) also showed that SARS-CoV-2 RNA is still detectable in sputum obtained by atomization from cured patients, although the pharynx swab test is negative. In addition, the viral load in the sputum is the highest compared to other specimens in the later stage of COVID-19 (Yoon et al., 2020). Therefore, it is necessary to collect lower respiratory tract specimens for SARS-CoV-2 RNA testing before COVID-19 patients can be discharged. Taken together, these results indicate that the sputum test was a more reliable criterion for discharge from hospital or quarantine release testing from a specimen taken from the upper airways. Moreover, since some patients (10%, such as patients #2 and #8) have positive feces longer than sputum, we recommend that the combined detection of feces and sputum is more reliable. Our data suggest that the presence of SARS-CoV-2 RNA in feces may be prolonged for more than a month in exceptional cases after a negative result in nasal-swab, throat-swab, and sputum samples. This observation is similar to that of Wu et al. (2020), who found that the positivity of SARS-CoV-2 RNA in fecal samples lagged behind that in respiratory samples. Currently, although no cases of SARS-CoV-2 transmission through fecal-oral route have been reported, the potential risk of fecal-oral transmission may be increased in closed residential spaces and areas with poor sanitary conditions. For themselves, SARS-CoV-2 can actively replicate in human intestinal organs, and infectious viruses can be isolated from fecal samples of COVID-19 patients (Zhou et al., 2020). Our results suggested that infected patients could potentially shed SARS-CoV-2 through respiratory and fecal-oral routes. Therefore, we suggest that RT-PCR should be employed to routine diagnosis of fecal samples after removal of SARS-CoV-2 RNA from the respiratory tract. A negative fecal result for SARS-CoV-2 nucleic acids in feces could be included in the rules for discharge from the hospital or lifting of quarantine measures as a supplement to sputum detection for patients recovering from COVID-19.

However, a negative result of viral nucleic acid cannot rule out the possibility of SARS-CoV-2 infection. We have further confirmed that combined detection of serum SARS-CoV-2 IgM and IgG was a practical and sensitive indicator, and also an effective complement to viral nucleic acid testing considering false-negative results upon it (Li et al., 2020). In addition, the duration of IgM/IgG-positivity from serum samples was much longer than that of SARS-CoV-2 nucleic acids from clinical samples. Serological test appears to be more meaningful for patients with an exposure history but who are negative for SARS-CoV-2 nucleic acids, regardless of whether the patients present symptoms or not. We speculate that serological test can effectively make up for the omission risk of viral nucleic acid detection, thus possessing important value in the timely diagnosis and prevention of COVID-19. Although nucleic acid test is difficult to avoid false negative, it can directly detect whether there is SARS-CoV-2 virus in human body, and its positive result is of great significance and is the gold standard for COVID-19 diagnosis (Shen et al., 2020). Compared with serological detection, nucleic acid detection can be applied in the early stage of SARS-CoV-2 infection, and indicate that it is now undergoing an infected state. However, in view of the high sensitivity of specific antibody detection, we suggest that it can be used as a supplementary indicator for nucleic acid detection, or even as a targeted remedy for leakage.

In summary, SARS-CoV-2 has multiple shedding ways and a more prolonged survival period in sputum specimens from COVID-19 patients. A comprehensive understanding of the viral shedding period in human body is extremely helpful to determine the time of release from quarantine or discharge from the hospital. We also recommend the application of a serological test to assist in identifying SARS-CoV-2 infection judged by viral nucleic acid test, especially when COVID-19-related symptoms have presented. Since COVID-19 has progress to one of the latest severe infectious diseases threatening human and restricting social activities worldwide, our findings

are critical for the diagnosis of SARS-CoV-2 infection and the prevention of virus transmission in convalescent COVID-19 patients.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary Material.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the First Affiliated Hospital of Guangzhou Medical University (Guangzhou, China). The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

WM and LW conceived and designed the study. JS conducted the experiments. JL analyzed the data. YH and JY wrote the paper. All authors reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the China National Science Foundation (grant no. 81503103).

ACKNOWLEDGMENTS

We thank the patients involved in the study. We acknowledge all healthcare workers involved in the diagnosis and treatment of patients with 2019 novel coronavirus disease in the First Affiliated Hospital of Guangzhou Medical University.

REFERENCES

Han, H. Q., Luo, Q. F., Mo, F., Long, L. M., and Zheng, W. Q. (2020). SARS-CoV-2 RNA more readily detected in induced sputum than in throat swabs of convalescent COVID-19 patients. *Lancet Infect. Dis.* 20, 655–656. doi: 10.1016/S1473-3099(20)30174-2

Huang, C. L., Wang, Y. M., Li, X. W., Ren, L. L., Zhao, J. P., Hu, Y., et al. (2020). Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet* 395, 497–506. doi: 10.1016/S0140-6736(20)30183-5

Li, D. S., Wang, D. W., Dong, J. P., Wang, N. N., Huang, H., Xu, H. W., et al. (2020). False-negative results of real-time reverse-transcriptase polymerase chain reaction for severe acute respiratory syndrome coronavirus 2: role of deep-learning-based CT diagnosis and insights from two cases. *Korean J. Radiol.* 21, 505–508. doi: 10.3348/kjr.2020.0146

Qi, X., Liu, C., Jiang, Z., Gu, Y., Zhang, G., Shao, C., et al. (2020). Multicenter analysis of clinical characteristics and outcomes in patients with COVID-19 who develop liver injury. *J. Hepatol.* 73, 455–458. doi: 10.1016/j.jhep.2020.04.010

Qu, Y. M., Kang, E. M., and Cong, H. Y. (2020). Positive result of Sars-Cov-2 in sputum from a cured patient with COVID-19. *Travel Med. Infect. Dis.* 34:101619. doi: 10.1016/j.tmaid.2020.101619

Rothe, C., Schunk, M., Sothmann, P., Bretzel, G., Froeschl, G., Wallrauch, C., et al. (2020). Transmission of 2019-nCoV infection from an asymptomatic contact in Germany. *N. Engl. J. Med.* 382, 970–971. doi: 10.1056/NEJMc2001468

Shen, M., Zhou, Y., Ye, J., Abdullah Al-Maskri, A. A., Kang, Y., Zeng, S., et al. (2020). Recent advances and perspectives of nucleic acid detection for coronavirus. *J. Pharm. Anal.* 10, 97–101. doi: 10.1016/j.jpha.2020.02.010

Tang, A., Tong, Z. D., Wang, H. L., Dai, Y. X., Li, K. F., Liu, J. N., et al. (2020). Detection of novel coronavirus by RT-PCR in stool specimen from asymptomatic child, China. *Emerg. Infect. Dis.* 26, 1337–1339. doi: 10.3201/eid2606.200301

Wölfel, R., Corman, V. M., Guggemos, W., Seilmaier, M., Zange, S., Müller, M. A., et al. (2020). Virological assessment of hospitalized patients with COVID-2019. *Nature* 581, 465–469. doi: 10.1038/s41586-020-2196-x

Wu, Y., Guo, C., Tang, L., Hong, Z., Zhou, J., Dong, X., et al. (2020). Prolonged presence of SARS-CoV-2 viral RNA in faecal samples. *Lancet Gastroenterol. Hepatol.* 5, 434–435. doi: 10.1016/S2468-1253(20)30083-2

Yoon, J. G., Yoon, J., Song, J. Y., Yoon, S. Y., Lim, C. S., Seong, H., et al. (2020). Clinical significance of a high SARS-CoV-2 viral load in the saliva. *J. Korean Med. Sci.* 35:e195. doi: 10.3346/jkms.2020.35.e195

Zhou, J., Li, C., Liu, X., Chiu, M. C., Zhao, X., Wang, D., et al. (2020). Infection of bat and human intestinal organoids by SARS-CoV-2. *Nat. Med.* 26, 1077–1083. doi: 10.1038/s41591-020-0912-6

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Medicine

ORIGINAL RESEARCH
published: 06 August 2020
doi: 10.3389/fmed.2020.00491

Clinical Characteristics and Short-Term Outcomes of Severe Patients With COVID-19 in Wuhan, China

Xiaobo Feng[1†], Peiyun Li[2†], Liang Ma[1†], Hang Liang[1], Jie Lei[1], Wenqiang Li[1], Kun Wang[1], Yu Song[1], Shuai Li[1], Wei Yang[3,4] and Cao Yang[1*]*

[1] Department of Orthopaedics, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China, [2] Department of Clinical Nutrition, Wuhan Children's Hospital (Wuhan Maternal and Child Healthcare Hospital), Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China, [3] Hubei Key Laboratory of Food Nutrition and Safety, Department of Nutrition and Food Hygiene, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China, [4] Department of Nutrition and Food Hygiene and MOE Key Lab of Environment and Health, School of Public Health, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China

OPEN ACCESS

Edited by:
Longxiang Su,
Peking Union Medical College Hospital (CAMS), China

Reviewed by:
Yanxing Wei,
University of Toronto, Canada
Guodong Fu,
Mount Sinai Hospital, University of Toronto, Canada

***Correspondence:**
Wei Yang
yw8278@hotmail.com;
yw8278@hust.edu.cn
Cao Yang
caoyangunion@hust.edu.cn

[†]These authors have contributed equally to this work and share first authorship

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: 20 May 2020
Accepted: 20 July 2020
Published: 06 August 2020

Citation:
Feng X, Li P, Ma L, Liang H, Lei J, Li W, Wang K, Song Y, Li S, Yang W and Yang C (2020) Clinical Characteristics and Short-Term Outcomes of Severe Patients With COVID-19 in Wuhan, China. Front. Med. 7:491. doi: 10.3389/fmed.2020.00491

Background: A novel pneumonia (COVID-19) spread rapidly throughout worldwide, in December, 2019. Most of the deaths have occurred in severe and critical cases, but information on prognostic risk factors for severely ill patients is incomplete. Further research is urgently needed to guide clinicians, and we therefore prospectively evaluate the clinical outcomes of 114 severely ill patients with COVID-19 for short-term at the Union Hospital in Wuhan, China.

Methods: In this single-centered, prospective, and observational study, we enrolled 114 severely ill patients with confirmed COVID-19 from Jan 23, 2020, to February 22, 2020. Epidemiological, demographic, laboratory, treatment, and outcome data were recorded, and the risk factors for poor outcome were analyzed.

Results: Among the 114 enrolled patients with a mean age of 63.96 ± 13.41 years, 94 (82.5%) patients were classified as a good outcome group. Common clinical manifestations included fever, cough, and fatigue. Compared with the good outcome group, 20 (17.5%) patients in the poor outcome group more frequently exhibited lymphopenia, and lower levels of albumin, partial arterial oxygen pressure, higher levels of lactate dehydrogenase, creatine kinase, hypersensitive troponin I, C-reactive protein, ferritin, blood urea nitrogen, and D-dimer, as well as markedly higher levels of IL-6 and IL-10. Absolute numbers of T lymphocytes, CD8 + T cells, decreased in almost all the patients and were markedly lower in the poor outcome group than the good outcome group. We also found that traditional Chinese medicine can significantly improve the patient's condition, which is conducive to the transformation from a severe to mild condition. In addition, univariate and multivariate Cox analyses of potential factors for poor outcome patients indicated that cytokine storms and uncontrolled inflammation responses as well as liver, kidney, and cardiac dysfunction are related to the development of a poor outcome.

Conclusion: In summary, we reported this single-centered, prospective, and observational study for short-term outcome in severe patients with COVID-19. We found that cytokine storms and uncontrolled inflammation responses as well as liver, kidney, and cardiac dysfunction may play important roles in the final outcome of severely ill patients with COVID-19. Our study will allow clinicians to benefit and rapidly estimate the likelihood of a short-term poor outcome for severely ill patients.

Keywords: COVID-19, SARS-CoV-2, severe patients, short-term outcomes, inflammation

INTRODUCTION

A pneumonia caused by a novel coronavirus, severe acute respiratory syndrome corona virus 2 (SARS-CoV-2), spread rapidly throughout worldwide in December 2019 (1). Despite progress made in our understanding of the characteristics of the disease, there are currently no drugs to combat SARS-CoV-2, and patients are primarily provided with supportive treatment. Several studies have indicated that the main symptoms of coronavirus disease 2019 (COVID-19) include fever, cough, and dyspnea (2–5). Huang et al. described the epidemiological, clinical, laboratory, and radiological characteristics of COVID-19, as well as various treatment strategies and outcomes, among 41 patients during the first wave of hospitalizations. They also compared clinical characteristics between patients treated in an intensive care unit (ICU) and those treated in non-ICU (2). Yang et al. also performed a detailed analysis of the patients critically ill with SARS-CoV-2 infection (5). In an analysis of 74 patients with COVID-19 exhibiting gastrointestinal symptoms, Jin et al. suggested that non-classical symptoms have been overlooked, posing a threat to the public (6). Wu et al. further noted that the risk of acute respiratory distress syndrome (ARDS) and death is increased in older adults (≥65 years old) with COVID-19 (7). Guo et al. found that diabetes is a risk factor for patients with COVID-19 (8). However, few prospective studies have explored the short-term outcomes of severely ill patients under current medical treatment and the risk factors that affect the short-term outcomes of severely ill patients, especially pneumonia patients with certain chronic diseases, which accounted for the majority of deaths. Here, we used a single-centered, prospective method to describe the basic clinical characteristics and short-term outcomes of severe patients in Union hospital, Wuhan, and we further aimed to explore the potential risk factors for poor outcomes among these patients using Cox proportional hazard models.

METHODS

Study Design and Participants

This single-center, prospective study included 114 severe patients with confirmed COVID-19 pneumonia hospitalized at the Union Hospital in Wuhan, China, which is a hospital designated to treat patients with COVID-19. From January 23, 2020, to February 22, 2020, we continuously enrolled patients diagnosed with COVID-19 based on interim guidance provided by the World Health Organization (WHO). Based on the Diagnosis and Treatment Scheme for SARS- CoV-2 of Chinese (The Seven Edition), severe patients were diagnosed if one or more of following criteria were met: dyspnea with respiratory rate (RR) ≥30 times/min, resting finger oxygen saturation ≤93%, and artery PaO_2/FiO_2 ≤300 mm Hg (1 mm Hg = 0.133 kPa). This study was approved by the Ethics Commission of Wuhan Union Hospital of Tongji Medical College, Huazhong University of Science and Technology. Written informed consent was waived due to the emergency of this infectious diseases.

Data Collection

Data related to clinical characteristics were collected using a case record form modified from the standardized International Severe Acute Respiratory and Emerging Infection Consortium case report form. Epidemiological and demographic data, including age, sex, and coexisting disorders, were also collected. The Baseline laboratory indices and radiographic findings were obtained from clinical electronic medical records. Moreover, the treatment strategies and outcomes were collected until the day of death /discharge or for the first 28 days after a diagnosis of severe illness, whichever was shorter. All missing or vague data, were obtained by communicating with patients and their families. All data were checked by two physicians (Xiaobo Feng and Liang Ma), and a third researcher (Wei Yang) adjudicated any difference in interpretation between the two primary reviewers.

Outcomes

Clinical outcomes after 28 days of consecutive observations were divided into two categories. Patients that had been discharged, those whose condition had been deemed non-severe, and those not requiring mechanical ventilation were considered to have experienced good outcomes. Patients requiring mechanical ventilation and those who had died were considered to have experienced poor outcomes. The criteria for discharge were as follows: normal temperature for more than 3 days ($T < 37.3^{\circ}C$), significant improvement in respiratory symptoms, pulmonary imaging showing significant improvement in acute exudative lesions, and nucleic acid tests negative for respiratory tract specimens such as sputum and nasopharyngeal swabs for two consecutive samplings (at least 24 h after sampling). Patients with mild clinical symptoms and no signs of pneumonia on radiography were considered to be non-severe. ARDS and shock were confirmed by the WHO guidance for COVID-19. Acute kidney injury was defined according to the serum creatinine. Cardiac injury was identified by the serum concentration of hypersensitive cardiac troponin I (hsTNI) and, if it was above the

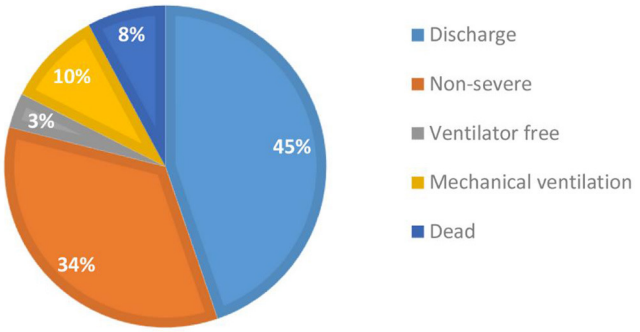


FIGURE 1 | Distribution of short-term (28 days) outcomes of severely ill patients with COVID-19 ($N = 114$).

upper limit of the reference range (>28 pg/mL), measured in the laboratory of Union Hospital (5).

Statistics

Continuous variables were expressed as means ± SDs if normally distributed and medians (IQRs) if skewed distributed while categorical variables were summarized as number (%). Differences between the characteristics of outcome groups were assessed using students *t*-test or Mann-Whitney U-test for continuous variables and chi-square tests for categorical variables. In addition, univariate and multivariate Cox proportional hazard models were used to determine hazard ratios (HRs) and 95% confidence intervals (CIs) of poor outcome in severe patients with COVID-19. The candidate risk factors included demographic and epidemiological characteristics as well as some laboratory indices. We determined the cut points of levels according to normal range, actual distribution, and clinical significance of each index. Adjustments were made for potential confounders, including age and sex. For risk factors identified in Cox analyses, we used restricted cubic spline model to further explore the potential dose-response relationship between factors and poor outcome risk. The referent (HR = 1) was set according to the cut point in Cox analyses. $P < 0.05$ was considered statistically significant. All data were analyzed using SPSS (23.0 IBM SPSS).

RESULTS

Clinical Outcomes

As of March 21, 2020, a total of 114 patients diagnosed with and treated for severe COVID-19 were enrolled in this study. Twenty-eight days after a diagnosis of severe COVID-19, good and poor outcomes were observed in 94 and 20 patients, respectively. As shown in **Figure 1**, 51 (45%) patients were alive and had been discharged, 39 (34%) had transitioned to non-severe illness, four (3%) remained severely ill but did not require a ventilator, i.e., severe status and ventilator free, 11 (10%) were alive but remained ventilated, and nine (8%) had died.

Demographics and Characteristics

The general demographic and epidemiological characteristics of all enrolled patients are summarized in **Table 1**. The mean age was 63.96 ± 13.41 years, 62 (54.4%) were older than 65 years and 71 (62.3%) were male. A total of 89 (78.1%) severe patients have a chronic medical illness, and the most coexisting disorders were hypertension with 62 (54.4%), diabetes with 39 (34.2%) and cardiovascular disease with 31 (27.2%). No significant differences were observed in such characteristics between outcome groups ($P \geq 0.05$ for all). **Table 2** displays the clinical characteristics of the patients. The duration from onset of symptoms to diagnosis of COVID-19 was 4.0 (2.0–7.0) days, while the duration from the onset of symptoms to the diagnosis of severe illness was 10.0 (6.0–14.3) days. For 114 patients, the most common symptoms at initial diagnosis were fever in 78 (68.4%), cough in 49 (43.0%), chest tightness in 34 (29.8%), and fatigue in 30 (26.3%) patients. Other symptoms, including shortness of breath in 18 (15.8%), anorexia in 12 (10.5%), chill in 12 (10.5%), myalgia in 10 (8.8%), sputum in nine (7.9%), headache in eight (7.0%), diarrhea in eight (7.0%), chest pain in three (2.6%), stomachache in three (2.6%) and nausea in three (2.6%) were relatively rare. As for oxygen saturation, patients in the poor outcome group had significantly lower levels than those in the good outcome group [median (IQR): 91 (90–93%) in the good outcome group and 81 (74–88%) in the bad outcome group, $P < 0.001$].

Laboratory and CT Findings

In terms of the 114 severe patients, many laboratory indicators differed significantly between outcome groups (**Table 3**). Compared with the good outcome group, the absolute counts of neutrophils 6.25 (4.69–9.20) vs. 3.48 (2.54–5.23), c-reactive protein 102.15 (78.07–122.90) vs. 48.95 (15.08–83.98), D dimer 2.10 (1.22–3.07) vs. 0.96 (0.41–.78), total bilirubin 19.20 (9.25–33.05) vs. 11.05 (8.53–14.05), blood urea nitrogen 9.02 (5.26–11.30) vs. 4.11 (3.11–5.04), creatine kinase 151.50 (50.50–218.50) vs. 62.00 (46.75–110.50), lactate dehydrogenase 638.00 (436.00–923.00) vs. 259.50 (213.75–382.50), hypersensitive cardiac troponin I 60.70 (18.48–298.98) vs. 4.10 (1.70–10.83), ferritin 679.00 (573.90–993.15) vs. 321.80 (231.00–532.88), interleukin-6 (IL-6) 76.10 (19.05–192.88) vs. 21.23 (7.23–47.61), and IL-10 6.59 (4.58–11.78) vs. 4.64 (3.65–6.18) were significantly higher in poor outcome. Besides, total protein 60.40 (56.78–64.05) vs. 63.80 (59.33–68.50) and PaO2 68.15 (49.00–77.75) vs. 81.00 (74.75–89.00) were significantly lower in poor outcome group. For chest X- ray/CT, 107 (93.9) patients had Ground-glass opacity. These data indicated that the uncontrolled inflammation responses, infection, liver, and kidney dysfunction, and hypoxia may contribute to poor outcomes in patients with COVID-19.

Complications and Treatments

As shown in **Table 4**, patients with severe COVID-19 had complications including acute liver injury, ARDS, acute kidney injury, arrhythmia, acute myocardial injury, Disseminated Intravascular Coagulation (DIC), rhabdomyolysis, and septic shock. However, no patients in the good outcome group experienced septic shock. All patients in the poor outcome group experienced ARDS. Acute myocardial injury, acute kidney

TABLE 1 | Demographic and epidemiological characteristics of severe patients with COVID-19.

	Total (*N* = 114)	Good outcome (*N* = 94)	Poor outcome (*N* = 20)	*P*
Age (years)	63.96 ± 13.41	62.85 ± 13.65	69.15 ± 11.08	0.056
<65	52 (45.6)	46 (48.9)	6 (30.0)	0.123
≥65	62 (54.4)	48 (51.1)	14 (70.0)	
Sex, male	71 (62.3)	58 (61.7)	13 (65.0)	0.782
Hospital infection	7 (6.1)	6 (6.4)	1 (5.0)	>0.999
Coexisting disorders	89 (78.1)	73 (77.7)	16 (80.0)	>0.999
Diabetes	39 (34.2)	34 (36.2)	5 (25.0)	0.339
Hypertension	62 (54.4)	50 (53.2)	12 (60.0)	0.579
Hyperlipidemia	17 (14.9)	15 (16.0)	2 (10.0)	0.739
Cardiovascular diseases	31 (27.2)	24 (25.5)	7 (35.0)	0.388
Cerebrovascular diseases	6 (5.3)	3 (3.2)	3 (15.0)	0.110
Cancer	10 (8.8)	9 (9.6)	1 (5.0)	0.825
Chronic renal diseases	6 (5.3)	4 (4.3)	2 (10.0)	0.622
Chronic liver diseases	4 (3.5)	3 (3.2)	1 (5.0)	0.543
Chronic Obstructive Pulmonary Disease	11 (9.6)	9 (9.6)	2 (10.0)	>0.999
Neuropsychiatric disorders	3 (2.6)	2 (2.1)	1 (5.0)	0.443
History of surgery	33 (28.9)	26 (27.7)	7 (35.0)	0.511

Values are n (%) for categorical data, means ± SDs for normally distributed data, or medians (IQRs) for non-normally distributed data.

injury, arrhythmia, rhabdomyolysis, and DIC were significantly higher than their counterparts in 13.8, 22.3, 17.0, 2.1, and 2.1% of patients with COVID-19 in the good outcome group, respectively. All 114 patients with severe COVID-19 were treated with antibiotics and high flow nasal cannula, while 25 (21.9%) were treated with non-invasive mechanical ventilation and 22 (19.3%) with invasive mechanical ventilation treatment. Six (5.3%) patients were treated with extracorporeal membrane oxygenation (ECMO) and all of whom were in the poor outcome group. Almost all [113 (99.1%)] patients received antiviral treatment, including arbidol hydrochloride capsules (0.2 g three times daily), lopinavir, and ribavirin (500 mg two times daily) via the oral route. Furthermore, as many as 41.2% patients received glucocorticoid therapy. Sixty-four (56.1%) patients received immunoglobulin treatment, and 49 (43.0%) patients were treated with parenteral nutrition; the percentage was higher in the poor outcome group than in the good outcome group [20 (100.0%) vs. 29 (30.9%)]. Two patients (1.8%) were treated with renal replacement therapy and 20 (17.5%) with vasoconstrictive agents, and it was higher than in the good outcome group [19 (95.0%) vs. 1 (1.1%)]. Moreover, the patients were given Traditional Chinese medicine (TCM) based on the protocol (9). All 20 patients in the poor group were transferred to the ICU, which was significantly higher than that of 9 (9.6%) in the good outcome group.

Prediction of Risk Factors for Severe COVID-19 in the Poor Outcome Group

Tables 5, **6** display the results of univariate and multivariate Cox analyses of potential risk factors for short-term outcomes in severe patients with COVID-19. Our results indicated that, for severe patients, higher levels of oxygen saturation (HR, 0.123; 95% CI, 0.041–0.369), albumin (HR, 0.060; 95% CI, 0.008–0.460), and arterial partial pressure of oxygen (HR, 0.321; 95% CI, 0.106–0.973) were associated with decreased risk of developing poor outcome within 28 days. In the other hand, higher levels of leucocytes (HR, 5.575; 95% CI, 2.080–14.943), neutrophils (HR, 2.566; 95% CI, 1.022–6.443), total bilirubin (HR, 6.171; 95% CI, 2.458–15.496), globulin (HR, 2.526; 95% CI, 1.027–6.211), blood urea nitrogen (HR, 5.640; 95% CI, 2.193–14.509), creatine kinase-MB (HR, 3.032; 95% CI, 1.203–7.644), lactate dehydrogenase (HR, 4.607; 95% CI, 1.057–20.090), hypersensitive cardiac troponin I (HR, 5.023; 95% CI, 1.921–13.136), lactate concentration (HR,15.721; 95% CI, 2.099–117.777), Interleukin-10 (HR, 3.551; 95% CI, 1.280–9.857), and C-reactive protein (HR, 5.275; 95% CI, 1.517–18.344) were associated with increased risk of poor outcome development. For all the factors analyzed above, increased concentration of lactate (≥1.6 mmol/L) and total bilirubin (≥19.0 μmol/L) might be the most important predictors of poor outcome in the early stage. As shown in **Figure 2**, non-linear dose-response relationship was also found between 10 indices and poor outcome risk in the cubic spline model.

DISCUSSION

The global spread of SARS-CoV-2 poses a significant threat to public health. Previous studies have shown that 20% of COVID-19 patients developed critical disease due to hypoxia or respiratory failure. Among them, 5% require treatment in the ICU, while 15% require oxygen and essential care. This suggests that this is particularly important in understanding this part of the patient (10). Recently, Dong et al. found that children, particularly infants, developed severe outcomes (11). This indicated that patients of any age could develop severe illness. Feng et al. found that severe and critical patients with the typical characteristics of multiple organ and immune function

TABLE 2 | Clinical characteristics of severe patients with COVID-19.

	Total (*N* = 114)	Good outcome (*N* = 94)	Poor outcome (*N* = 20)	*P*
Onset of symptom to, d				
Diagnosis	4.0 (2.0–7.0)	4.0 (2.0–7.0)	4.5 (2.3–10.8)	0.517
Serious illness	10.0 (6.0–14.3)	10.0 (6.0–15.0)	8.0 (5.0–14.0)	0.540
Signs and symptoms at initial				
Fever	78 (68.4)	63 (67.0)	15 (75.0)	0.486
Chest tightness	34 (29.8)	27 (28.7)	7 (35.0)	0.577
Shortness of breath	18 (15.8)	13 (13.8)	5 (25.0)	0.365
Cough	49 (43.0)	39 (41.5)	10 (50.0)	0.485
Sputum	9 (7.9)	8 (8.5)	1 (5.0)	0.943
Fatigue	30 (26.3)	28 (29.8)	2 (10.0)	0.068
Headache	8 (7.0)	7 (7.4)	1 (5.0)	>0.999
Myalgia	10 (8.8)	8 (8.5)	2 (10.0)	>0.999
Chest pain	3 (2.6)	3 (3.2)	0 (0.0)	1.000
Anorexia	12 (10.5)	12 (12.8)	0 (0.0)	0.198
Chill	12 (10.5)	8 (8.5)	4 (20.0)	0.263
Stomachache	3 (2.6)	3 (3.2)	0 (0.0)	>0.999
Diarrhea	8 (7.0)	7 (7.4)	1 (5.0)	>0.999
Nausea	3 (2.6)	2 (2.1)	1 (5.0)	0.443
Temperature at disease onset (°C)	38.1 (36.7–38.8)	38.1 (36.7–38.7)	38.2 (37.0–39.0)	0.561
<37.4	36 (31.6)	31 (33.0)	5 (25.0)	0.622
37.4–39.0	63 (55.3)	50 (53.2)	13 (65.0)	
>39.0	15 (13.2)	13 (13.8)	2 (10.0)	
Signs and symptoms at hospital admission				
Fever	32 (28.1)	25 (26.6)	7 (35.0)	0.448
Chest tightness	50 (43.9)	46 (48.9)	4 (20.0)	0.018
Shortness of breath	68 (59.6)	49 (52.1)	19 (95.0)	<0.001
Cough	9 (7.9)	7 (7.4)	2 (10.0)	>0.999
Fatigue	43 (37.7)	37 (39.4)	6 (30.0)	0.433
Headache	6 (5.3)	6 (6.4)	0 (0.0)	0.542
Myalgia	5 (4.4)	5 (5.3)	0 (0.0)	0.585
Chest pain	3 (2.6)	3 (3.2)	0 (0.0)	>0.999
Anorexia	18 (15.8)	16 (17.0)	2 (10.0)	0.657
Diarrhea	10 (8.8)	7 (7.4)	3 (15.0)	0.516
Nausea	2 (1.8)	2 (2.1)	0 (0.0)	>0.999
General signs at admission				
Temperature (°C)	36.7 (36.4–37.4)	36.7 (36.4–37.3)	38.2 (37.0–39.0)	0.279
<37.4	85 (74.6)	72 (76.6)	13 (65.0)	0.535
37.4–39.0	26 (22.8)	20 (21.3)	6 (30.0)	
>39.0	3 (2.6)	2 (2.1)	1 (5.0)	
Heart rate (/min)	86 (78–102)	87 (78–102)	88 (78–102)	0.456
<100	83 (72.8)	69 (73.4)	14 (70.0)	0.756
>100	31 (27.2)	25 (26.6)	6 (30.0)	
Respiratory rate (/min)	20 (20–23)	20 (20–22)	21 (20–30)	0.122
<20	22 (19.3)	19 (20.2)	3 (15.0)	0.760
≥20	92 (80.7)	75 (79.8)	17 (85.0)	
Oxygen saturation (%)	90 (88–92)	91 (90–93)	81 (74–88)	<0.001
<90	35 (30.7)	19 (20.2)	16 (80.0)	<0.001
≥90	79 (69.3)	75 (79.8)	4 (20.0)	

Values are n (%) for categorical data, means ± SDs for normally distributed data, or medians (IQRs) for non-normally distributed data.

TABLE 3 | Laboratory and radiographic findings at baseline of severe patients with COVID-19.

	Total (*N* = 114)	Good outcome (*N* = 94)	Poor outcome (*N* = 20)	*P*
Hematologic				
Leucocytes ($\times 10^9$ /L)	6.28 ± 3.36	5.74 ± 2.49	8.78 ± 5.36	0.022
Neutrophils ($\times 10^9$ /L)	3.88 (2.65–5.81)	3.48 (2.54–5.23)	6.25 (4.69–9.20)	<0.001
Lymphocytes ($\times 10^9$ /L)	0.87 (0.65–1.31)	0.92 (0.70–1.43)	0.67 (0.43–0.89)	0.001
Monocytes ($\times 10^9$ /L)	0.44 (0.30–0.59)	0.48 (0.33–0.61)	0.34 (0.22–0.40)	0.009
Platelets ($\times 10^9$ /L)	192.00 (140.74–269.75)	205.00 (142.75–272.50)	165.00 (138.25–218.00)	0.160
Hemoglobin (g/L)	122.04 ± 17.78	122.65 ± 16.75	119.15 ± 22.29	0.427
CD4+ T cells (%)	43.54 (36.45–53.41)	44.98 (36.53–53.20)	39.95 (31.46–57.32)	0.687
CD8+ T cells (%)	21.53 (16.48–28.55)	22.11 (17.61–29.95)	17.90 (14.00–23.45)	0.025
Coagulation function				
Prothrombin time (s)	13.5 (12.8–14.3)	13.40 (12.70–14.23)	14.25 (12.93–15.28)	0.072
Activated partial thromboplastin time (s)	38.00 (35.18–41.53)	38.00 (35.10–41.43)	38.45 (35.45–43.98)	0.398
Fibrinogen (g/L)	4.98 ± 1.48	5.05 ± 1.43	4.66 ± 1.67	0.282
Thrombin time (s)	17.35 (16.58–18.30)	17.35 (16.77–18.15)	17.20 (15.35–19.18)	0.467
D-dimer (mg/L)	1.06 (0.51–2.10)	0.96 (0.41–1.78)	2.10 (1.22–3.07)	0.005
Biochemical				
Alanine aminotransferase (U/L)	45.50 (26.00–74.25)	44.50 (25.75–72.50)	49.00 (29.25–80.25)	0.427
Aspartate aminotransferase (U/L)	39.50 (26.75–64.50)	38.50 (26.00–57.25)	44.00 (29.50–114.75)	0.197
Total bilirubin (μmol/L)	11.35 (8.93–16.15)	11.05 (8.53–14.05)	19.20 (9.25–33.05)	0.024
Total protein (g/L)	63.10 (58.48–67.70)	63.80 (59.33–68.50)	60.40 (56.78–64.05)	0.015
Albumin (g/L)	34.65 (30.350–38.60)	35.80 (31.85–39.10)	30.05 (27.10–32.65)	<0.001
Globulin (g/L)	29.15 (25.70–31.65)	28.95 (25.68–30.78)	30.35 (29.63–35.50)	0.057
Prealbumin (mg/L)	120 (90–153)	120 (90–151)	93 (82–181)	0.145
Blood urea nitrogen (mmol/L)	4.39 (3.27–6.13)	4.11 (3.11–5.04)	9.02 (5.26–11.30)	<0.001
Creatinine (μmol/L)	74.35 (60.00–87.85)	74.35 (60.28–86.38)	71.80 (53.70–95.60)	0.636
Creatine kinase (U/L)	66.50 (46.75–133.50)	62.00 (46.75–110.50)	151.50 (50.50–218.50)	0.046
Creatine kinase-MB (U/L)	17.00 (14.00–23.00)	17.00 (14.00–22.00)	19.50 (13.00–31.75)	0.234
Lactate dehydrogenase (U/L)	286.00 (223.75–452.25)	259.50 (213.75–382.50)	638.00 (436.00–923.00)	<0.001
Hypersensitive cardiac troponin I (ng/L)	5.70 (2.10–19.05)	4.10 (1.70–10.83)	60.70 (18.48–298.98)	<0.001
Glucose (mmol/L)	6.20 (5.20–8.31)	6.13 (5.16–7.63)	8.26 (5.81–13.42)	0.013
Serum potassium (mmol/L)	4.03 (3.70–4.50)	4.07 (3.72–4.51)	3.97 (3.59–4.36)	0.344
Serum sodium (mmol/L)	138.85 (136.30–142.00)	138.20 (136.00–141.68)	142.40 (138.85–146.93)	0.001
Serum calcium (mmol/L)	2.15 ± 0.18	2.17 ± 0.16	2.04 ± 0.20	0.003
Serum phosphorus (mmol/L)	1.00 (0.88–1.11)	1.00 (0.89–1.11)	0.95 (0.73–1.12)	0.381
Serum chlorine (mmol/L)	100.48 ± 5.10	100.03 ± 4.45	102.61 ± 7.20	0.137
Lactate concentration (mmol/L)	1.60 (1.38–1.80)	1.50 (1.30–1.70)	2.10 (1.70–2.40)	<0.001
Positive Urinary protein, n	47 (41.2)	33 (35.1)	14 (70.0)	0.004
Positive Urinary glucose, n	8 (7.0)	7 (7.4)	1 (5.0)	>0.999
Positive urinary occult blood, n	31 (27.2)	21 (22.3)	10 (50.0)	0.012
Blood gas characteristics				
pH	7.39 (7.36–7.42)	7.38 (7.36–7.42)	7.41 (7.31–7.46)	0.636
Arterial partial pressure of oxygen (mm Hg)	79.00 (70.00–88.00)	81.00 (74.75–89.00)	68.15 (49.00–77.75)	<0.001
Arterial partial pressure of carbon dioxide (mm Hg)	43.00 (38.00–46.00)	42.80 (38.00–45.00)	45.50 (35.60–57.00)	0.198
Infection-related biomarkers				
Interleukin 2 (pg/mL)	2.70 (2.43–3.04)	2.70 (2.47–3.02)	2.69 (2.41–3.60)	0.991
Interleukin 4 (pg/mL)	2.16 (1.85–2.60)	2.16 (1.89–2.52)	2.21 (1.68–3.50)	0.729

(Continued)

TABLE 3 | Continued

	Total (*N* = 114)	Good outcome (*N* = 94)	Poor outcome (*N* = 20)	*P*
Interleukin 6 (pg/mL)	23.28 (8.31–54.23)	21.23 (7.23–47.61)	76.10 (19.05–192.88)	0.002
Interleukin 10 (pg/mL)	4.91 (3.92–6.74)	4.64 (3.65–6.18)	6.59 (4.58–11.78)	0.001
Tumor necrosis factor-α (pg/mL)	2.63 (2.11–4.80)	2.63 (2.11–4.80)	2.67 (2.07–4.66)	0.838
Interferon-γ (pg/mL)	2.50 (1.96–3.20)	2.46 (1.96–3.20)	2.58 (1.89–3.49)	0.571
C-reactive protein (mg/L)	67.95 (20.50–103.25)	48.95 (15.08–83.98)	102.15 (78.07–122.90)	<0.001
Ferritin (ng/mL)	390.60 (261.70–721.00)	321.80 (231.00–532.88)	679.00 (573.90–993.15)	0.001
IgM, *n*	60 (68.2)	52 (69.3)	8 (61.5)	0.748
IgG, *n*	88 (100.0)	75 (100.0)	13 (100.0)	NA
Chest X- ray/CT findings				
Ground-glass opacity	107 (93.9)	87 (92.6)	20 (100.0)	0.351
Unilateral pneumonia	11 (9.6)	11 (11.7)	0 (0.0)	0.208
Bilateral pneumonia	102 (89.5)	82 (87.2)	20 (100.0)	0.122
Interstitial abnormalities	21 (18.4)	15 (16.0)	6 (30.0)	0.200

Data are n (%), means ± SDs or medians (IQRs) when appropriate.

TABLE 4 | Complications and treatments of severe patients with COVID-19.

	Total (*N* = 114)	Good outcome (*N* = 94)	Poor outcome (*N* = 20)	*P*
Complications				
Shock	8 (7.0)	0 (0.0)	8 (40.0)	<0.001
Acute respiratory distress syndrome	41 (36.0)	21 (22.3)	20 (100.0)	<0.001
Acute renal injury	35 (30.7)	21 (22.3)	14 (70.0)	<0.001
Acute myocardial injury	28 (24.6)	13 (13.8)	15 (75.0)	<0.001
Acute liver function injury	69 (60.5)	57 (60.6)	12 (60.0)	0.958
Arrhythmia	31 (27.2)	16 (17.0)	15 (75.0)	<0.001
Rhabdomyolysis	11 (9.6)	2 (2.1)	9 (45.0)	<0.001
Disseminated intravascular coagulation	15 (13.2)	2 (2.1)	13 (65.0)	<0.001
Treatment				
Antibiotic treatment	114 (100.0)	94 (100.0)	20 (100.0)	NA
Anticoronavirus treatment	113 (99.1)	93 (98.9)	20 (100.0)	>0.999
Glucocorticoids	47 (41.2)	28 (29.8)	19 (95.0)	<0.001
Oxygen therapy	114 (100.0)	94 (100.0)	20 (100.0)	NA
Immunoglobulin	64 (56.1)	45 (47.9)	19 (95.0)	<0.001
Parenteral nutrition	49 (43.0)	29 (30.9)	20 (100.0)	<0.001
Admission to intensive care unit	29 (25.4)	9 (9.6)	20 (100.0)	<0.001
Non-invasive ventilation	25 (21.9)	13 (13.8)	12 (60.0)	<0.001
Invasive mechanical ventilation	22 (19.3)	4 (4.3)	18 (90.0)	<0.001
Extracorporeal membrane oxygenation	6 (5.3)	0 (0.0)	6 (30.0)	<0.001
Vasoconstrictive agents	20 (17.5)	1 (1.1)	19 (95.0)	<0.001
Renal replacement therapy	2 (1.8)	0 (0.0)	2 (10.0)	0.029
Traditional Chinese medicine	86 (75.4)	77 (81.9)	9 (45.0)	0.001
Trastuzumab	13 (11.4)	12 (12.8)	1 (5.0)	0.459
Infusions of blood plasma	4 (3.5)	2 (2.1)	2 (10.0)	0.141
Onset of severe illness to, d*				
1st for RT-PCR (–)	14.0 (11.0–18.0)	14.0 (10.0–18.0)	19.0 (15.5–24.0)	<0.001
2nd for RT-PCR (–)	17.5 (14.0–22.0)	17.0 (14.0–21.5)	24.0 (22.0–27.5)	<0.001

Data are n (%) or medians (IQRs) when appropriate.
**Data available for 98 patients.*

TABLE 5 | Univariate and multivariate analyses of potential factors (demographic and epidemiologic) predicting poor outcome.

Factors	Level	Crude HR (95% CI)	*P*	Adjusted HR (95% CI)*	*P*
Age (years)	≥65 vs. <65	2.192 (0.842–5.708)	0.108	2.184 (0.839–5.687)	0.110
Sex	Female vs. Male	0.772 (0.308–1.937)	0.581	0.732 (0.292–1.838)	0.507
Coexisting disorders	Yes vs. No	1.154 (0.386–3.453)	0.797	0.692 (0.207–2.313)	0.550
Diabetes	Yes vs. No	0.706 (0.257–1.945)	0.380	0.622 (0.224–1.728)	0.363
Hypertension	Yes vs. No	1.122 (0.458–2.747)	0.801	0.960 (0.386–2.384)	0.929
Hyperlipidemia	Yes vs. No	0.677 (0.157–2.919)	0.601	0.729 (0.168–3.167)	0.673
Cardiovascular diseases	Yes vs. No	1.601 (0.638–4.015)	0.316	1.062 (0.380–2.970)	0.908
Cerebrovascular diseases	Yes vs. No	3.327 (0.975–11.356)	0.055	2.326 (0.612–8.848)	0.216
Cancer	Yes vs. No	0.536 (0.072–4.004)	0.543	0.410 (0.054–3.103)	0.388
Chronic renal diseases	Yes vs. No	3.678 (0.835–16.202)	0.085	3.437 (0.764–15.465)	0.108
Chronic liver diseases	Yes vs. No	1.433 (0.192–10.707)	0.726	0.997 (0.128–7.760)	0.997
Chronic Obstructive Pulmonary Disease	Yes vs. No	0.991 (0.230–4.272)	0.990	0.642 (0.139–2.955)	0.569
Neuropsychiatric disorders	Yes vs. No	1.186 (0.158–8.894)	0.868	0.734 (0.094–5.741)	0.768
History of surgery	Yes vs. No	1.168 (0.466–2.930)	0.740	1.041 (0.413–2.623)	0.932
Temperature at disease onset (°C)	37.4–39.0 vs. <37.4	1.514 (0.540–4.248)	0.430	1.844 (0.638–5.326)	0.258
	>39.0 vs. <37.4	1.036 (0.201–5.342)	0.966	2.714 (0.348–21.190)	0.622
Temperature at admission (°C)	37.4–39.0 vs. <37.4	1.476 (0.561–3.885)	0.430	1.721 (0.646–4.580)	0.277
	>39.0 vs. <37.4	2.630 (0.343–20.167)	0.352	2.714 (0.348–21.190)	0.341
Respiratory rate (/min)	≥20 vs. <20	1.357 (0.398–4.629)	0.626	1.316 (0.385–4.502)	0.662
Oxygen saturation (%)	≥90 vs. <90	0.131 (0.044–0.394)	<0.001	0.123 (0.041–0.369)	<0.001

**Adjustments were made for age and sex.*

dysfunction. They also found that older people aged ≥ 75 years are a risk factor for mortality (12). With the increase number of asymptomatic infectious patients, taking measures to detect and isolate early are especially important. In our study, we used a short-term method to prospectively study the reported the epidemiology and risk factors of 114 severe patients with COVID-19 from the Union hospital, Hubei province. To our knowledge, this is the first report to describes the severe patients with COVID-19 during a short-term observation and predict some risk factors for final outcome. In our study, the mean age of severe patients were 63.96 ± 13.41 years, and 58 (50.9%) were older than 65 years; the patients are thus older than in other studies (5, 13, 14). We also found that 78 (68.4%) of 114 patients initially exhibited fever, in accordance with previous studies, where fever is the one of the most common symptom in patients who had COVID-19 (5, 14–16). But, 36 (31.6%) of 114 severe patients did not exhibit fever at the beginning of illness, and other clinical manifestations should therefore be concerned. Recently, Jin et al. found that attention should also be paid to people who have gastrointestinal symptoms (6). Mao et al. indicated that clinicians should suspect COVID-19 also in patients with neurological manifestations (14).

According to results from laboratory tests, the poor outcome group had lower lymphocytes than the good outcome group [0.67 (0.43–0.89) vs. 0.92 (0.70–1.43)]. As is known to all, lymphocytes are the main fighting force against the virus, and we suspected that SARS-CoV-2 damages the lymphocyte and causes its reduction (17). Chen et al. found that severe lymphopenia were persistent and we more increased in dead patients than recovered patients, and they suggested that lymphopenia may be associated with poor outcome (18). Tan et al. demonstrated a contrasting result: lymphopenia is an effective indicator for the severity of patients with COVID-19 (19). CD8+ T cells were significantly lower in the poor outcome group. Chen et al. indicated that the SARS-CoV-2 infection may affect CD4+ and CD8+ T lymphocyte cells in particular and argue that this is a potential correlation with COVID-19 severity (20). In addition, markedly higher concentrations of cardiac troponin I, creatine kinase, and lactate dehydrogenase could be observed in the poor outcome group than in their counterpart. Most notably, patients who exhibited a poor outcome may develop pulmonary and extra-pulmonary organ damage, including septic shock, acute respiratory distress syndrome, acute kidney injury, acute cardiac injury, as well as disseminated intravascular coagulation. The fatality risk of COVID-19 patients with or without a history of previous cardiovascular disease may include acute cardiac injury and heart failure (18). Costanza Emanueli et al. suggested that the COVID-19 crisis will have long-term residual repercussions on the cardiovascular system (21). The suggestion is that the cardiac injury also requires special attention. In our study, we also found that lactate concentration was higher in poor outcomes than their counterpart. Lactate is generally the end product of energy through anaerobic metabolism, and the elevation of lactate levels is mainly caused by the increase of blood oxygen deficiency and anaerobic metabolism; this result is consistent with the lower oxygen saturation in the poor group, and this indicated that lactate level is an important predictor of poor outcome in the early stage. In addition, total bilirubin was also an important predictor of poor outcome in the early stage. Qi recommend that dynamic monitoring of the liver function of

TABLE 6 | Univariate and multivariate analyses of potential factors (laboratory indexes) predicting poor outcome.

Factors	Normal range	Level†	Crude HR (95% CI)	*P*	Adjusted HR (95% CI)*	*P*
Hematologic						
Leucocytes ($\times 10^9$ /L)	3.5–9.5	≥9.5 vs. <9.5	4.634 (1.840–11.669)	0.001	5.575 (2.080–14.943)	0.001
Neutrophils ($\times 10^9$ /L)	1.8–6.3	≥6.3 vs. <6.3	2.663 (1.102–6.433)	0.030	2.566 (1.022–6.443)	0.045
Lymphocytes ($\times 10^9$ /L)	1.1–3.2	≥1.1 vs. <1.1	0.293 (0.068–1.266)	0.100	0.337 (0.077–1.475)	0.149
Monocytes ($\times 10^9$ /L)	0.1–0.6	≥0.6 vs. <0.6	0.179 (0.024–1.335)	0.179	0.182 (0.024–1.366)	0.098
Platelets ($\times 10^9$ /L)	125.0–350.0	≥125.0 vs. <125.0	0.733 (0.245–2.192)	0.578	0.837 (0.275–2.553)	0.755
Hemoglobin (g/L)	130.0–175.0	≥130.0 vs. <130.0	0.865 (0.354–2.116)	0.751	0.652 (0.244–1.732)	0.394
CD4+ T cells (%)	25.34–51.37	≥51.37 vs. <51.37	1.144 (0.439–2.980)	0.783	1.235 (0.468–3.259)	0.670
CD8+ T cells (%)	14.23–38.95	≥14.23 vs. <14.23	0.687 (0.249–1.890)	0.467	0.867 (0.303–2.482)	0.790
Coagulation function						
Prothrombin time (s)	11.0–16.0	≥13.5 vs. <13.5	1.574 (0.644–3.852)	0.320	1.112 (0.417–2.966)	0.832
Activated partial thromboplastin time (s)	28.0–43.5	≥43.5 vs. <43.5	1.363 (0.493–3.766)	0.551	1.204 (0.433–3.344)	0.722
Fibrinogen (g/L)	2.0–4.0	≥4.0 vs. <4.0	0.533 (0.212–1.338)	0.180	0.520 (0.205–1.317)	0.168
Thrombin time (s)	14.0–21.0	≥17.4 vs. <17.4	1.114 (0.463–2.678)	0.809	1.197 (0.489–2.930)	0.694
D-dimer (mg/L)	<0.5	≥0.5 vs. <0.5	1.232 (0.411–3.697)	0.710	0.940 (0.294–3.001)	0.917
Biochemical						
Alanine aminotransferase (U/L)	5–40	≥40 vs. <40	0.961 (0.393–2.351)	0.931	1.203 (0.429–3.373)	0.726
Aspartate aminotransferase (U/L)	8–40	≥40 vs. <40	1.611 (0.658–3.942)	0.296	1.900 (0.755–4.783)	0.173
Total bilirubin (μmol/L)	5.1–19.0	≥19.0 vs. <19.0	5.849 (2.433–14.063)	<0.001	6.171 (2.458–15.496)	<0.001
Total protein (g/L)	60–80	≥60 vs. <60	0.687 (0.284–1.661)	0.405	0.721 (0.298–1.748)	0.470
Albumin (g/L)	35–55	≥35 vs. <35	0.054 (0.007–0.405)	0.054	0.060 (0.008–0.460)	0.007
Globulin (g/L)	20–30	≥30 vs. <30	2.723 (1.113–6.666)	0.028	2.526 (1.027–6.211)	0.043
Prealbumin (mg/L)	170–420	≥170 vs. <170	1.001 (0.364–2.755)	0.999	1.282 (0.448–3.665)	0.643
Blood urea nitrogen (mmol/L)	2.9–8.2	≥8.2 vs. <8.2	6.283 (2.565–15.391)	<0.001	5.640 (2.193–14.509)	<0.001
Creatinine (μmol/L)	44–133	≥74 vs. <74	0.973 (0.405–2.337)	0.950	0.709 (0.254–1.978)	0.511
Creatine kinase (U/L)	38–174	≥174 vs. <174	2.039 (0.783–5.307)	0.144	1.982 (0.756–5.199)	0.164
Creatine kinase-MB (U/L)	0–24	≥24 vs. <24	2.449 (1.000–5.997)	0.050	3.032 (1.203–7.644)	0.019
Lactate dehydrogenase (U/L)	109–245	≥245 vs. <245	3.963 (0.915–17.161)	0.066	4.607 (1.057–20.090)	0.042
Hypersensitive cardiac troponin I (ng/L)	<26.2	≥26.2 vs. <26.2	5.613 (2.233–14.112)	<0.001	5.023 (1.921–13.136)	0.001
Glucose (mmol/L)	3.9–6.1	≥6.1 vs. <6.1	1.678 (0.637–4.416)	0.295	1.454 (0.543–3.893)	0.457
Serum potassium (mmol/L)	3.5–5.2	≥4.0 vs. <4.0	1.112 (0.462–2.677)	0.813	0.921 (0.368–2.304)	0.860
Serum sodium (mmol/L)	136–145	≥136 vs. <136	1.881 (0.436–8.120)	0.397	3.302 (0.702–15.535)	0.131
Serum calcium (mmol/L)	2.03–2.54	≥2.03 vs. <2.03	0.385 (0.160–0.925)	0.033	0.433 (0.173–1.083)	0.073
Serum phosphorus (mmol/L)	0.96–1.62	≥0.96 vs. <0.96	0.831 (0.345–2.001)	0.680	0.794 (0.330–1.914)	0.608
Serum chlorine (mmol/L)	96–108	≥96 vs. <96	1.617 (0.375–6.970)	0.519	2.195 (0.489–9.845)	0.305
Lactate concentration (mmol/L)	0.5–1.6	≥1.6 vs. <1.6	15.457 (2.067–115.615)	0.008	15.721 (2.099–117.777)	0.007
Positive Urinary protein	/	/	3.239 (1.244–8.433)	0.016	2.905 (1.099–7.678)	0.032
Positive Urinary glucose	/	/	0.893 (0.120–6.676)	0.912	0.961 (0.128–7.238)	0.969
Positive urinary occult blood	/	/	2.474 (1.030–5.945)	0.043	2.247 (0.932–5.421)	0.071
Blood gas characteristics						
pH	7.35–7.45	≥7.35 vs. <7.35	0.427 (0.170–1.074)	0.071	0.468 (0.181–1.207)	0.116
Arterial partial pressure of oxygen (mm Hg)	80–100	≥80 vs. <80	0.295 (0.098–0.883)	0.029	0.321 (0.106–0.973)	0.045
Arterial partial pressure of carbon dioxide (mm Hg)	35–45	≥45 vs. <45	2.159 (0.892–5.230)	0.088	2.224 (0.895–5.525)	0.085
Infection-related biomarkers						
Interleukin-2 (pg/mL)	0.1–4.1	≥4.1 vs. <4.1	1.820 (0.533–6.212)	0.339	2.343 (0.654–8.389)	0.191
Interleukin-4 (pg/mL)	0.1–3.2	≥3.2 vs. <3.2	1.663 (0.663–4.172)	0.279	2.112 (0.797–5.597)	0.133
Interleukin-6 (pg/mL)	0.1–2.9	≥23.3 vs. <23.3	1.782 (0.711–4.468)	0.218	1.485 (0.575–3.836)	0.414
Interleukin-10 (pg/mL)	0.1–5.0	≥5.0 vs. <5.0	2.629 (1.010–6.843)	0.048	3.551 (1.280–9.857)	0.015
Tumor necrosis factor-α (pg/mL)	0.1–23.0	≥2.6 vs. <2.6	1.079 (0.448–2.600)	0.866	1.032 (0.427–2.491)	0.945
Interferon-γ (pg/mL)	0.1–18.0	≥2.5 vs. <2.5	1.260 (0.522–3.043)	0.607	1.421 (0.582–3.473)	0.440
C-reactive protein (mg/L)	<8.0	≥65.0 vs. <65.0	4.703 (1.374–16.093)	0.014	5.275 (1.517–18.344)	0.009
Ferritin (ng/mL)	4.6–204.0	≥204.0 vs. <204.0	2.582 (0.210–11.930)	0.657	2.647 (0.311–22.522)	0.373

*****Adjustments were made for age and sex.*

† Cut points of levels were determined according to normal range, actual distribution, and clinical significance.

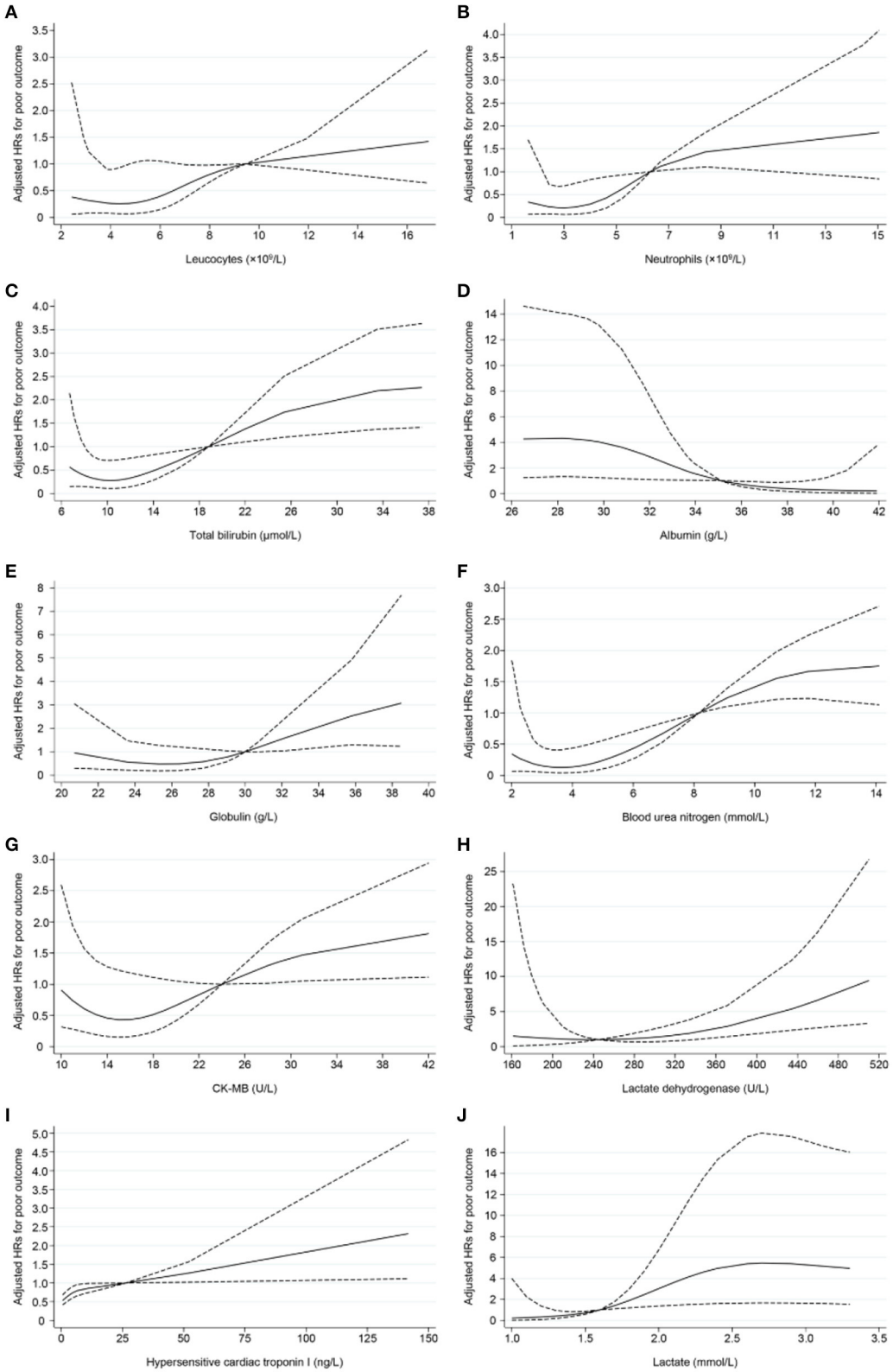


FIGURE 2 | Non-linear dose–response relationship between 10 indices and poor outcome risk. Hazard ratios (HRs) were adjusted for age and gender. Dotted lines represent the 95% CIs for the fitted trend. **(A)** Leucocytes ($\times 10^9$ /L), referent (HR = 1): 9.5; **(B)** Neutrophils ($\times 10^9$ /L), referent: 6.3; **(C)** Total bilirubin (μmol/L), referent: 19.0; **(D)** Albumin, referent: 35.0 (g/L); **(E)** Globulin (g/L), referent: 30.0; **(F)** Blood urea nitrogen (mmol/L), referent: 8.2; **(G)** CK-MB (U/L), referent: 24.0; **(H)** Lactate dehydrogenase (U/L), referent: 245; **(I)** Hypersensitive cardiac troponin I (ng/L), referent: 26.2; **(J)** Lactate (mmol/L), referent: 1.6.

patients is necessary (22). Cai et al. conclude that patients with abnormal liver function may had higher risks of progressing to severe disease (23). Due to the "cytokine storm" also observed in the poor outcome group, 19 (95.0%) of these patients were given glucocorticoid therapy. Wu et al. previously found that the administration of methylprednisolone may have reduced the risk of death in patients with ARDS (7). To our surprise, most of the severe patients treated with Traditional Chinese medicine (TCM) were eventually converted to a good outcome, indicating the importance of this effort on COVID-19. A large of clinical practice results indicated that TCM shows significant role in the patients with COVID-19. For the severe patients in the treatment of TCM, the mean length in hospital and the time of nucleic acid turning negative has been shortened by more than 2 days (24). Yang et al. analyzed the effect of Lian Hua Qing Wen Capsules in the treatment of COVID-19 patients, and they found that this TCM could markedly relieve fever and cough and promote recovery (25). Besides, a comprehensive evaluation and further scientific research should be carried out on the effect of TCM on COVID-19.

Meantime, the risk factors related to the poor outcome included uncontrolled inflammation responses, infection, hypoxia, and liver, kidney, and cardiac dysfunction. The pathogenesis of COVID-19 is still being studied. Cytokine storms and uncontrolled inflammation responses are thought to play important roles in the outcome of COVID-19 (26–30). External stimuli resulted in an excessive immune response, and the pathogenesis of the cytokine storm is complex and can leaded to rapid disease progression and high mortality. The inflammatory cytokine storm is closely correlated to the development and progression of ARDS (31). Neutrophils play important role in chemokines and cytokines (32). In our study, the poor outcome group had significantly higher neutrophil counts than the good outcome group, and this may be the underlying cause of the cytokine storm. In addition, $CD8^+$ T cells were significantly lower in the poor outcome group. These results highlight the important roles of $CD8^+$ T cells in COVID-19. Studies had shown that T cells could inhibit the over-activation of innate immunity (33). T cells can help to clear SARS-CoV, and a low T-cell response can result in pathological changes in mice with SARS-CoV (34). The relevant mechanisms need to be studied further.

This study has some limitations. First, owing to the limited number of cases, only 114 severe patients were included. Second, this study was a single-center research, and a larger cohort study of severe patients with SARS-CoV-2 from other cities in China and other countries would help to further describe the clinical characteristics and predict risk factors related to this disease. Third, although we included numerous factors that may be associated with clinical outcome in the analyses and made adjustment for potential confounders when exploring the associations, we could not rule out the possibility of other residual confounders.

In summary, the present study is a single-center, prospective observational study that examined clinical characteristics and risk factors for poor short-term outcomes in patients with severe COVID-19. Our univariate and multivariate analyses demonstrated that cytokine storm/uncontrolled inflammatory responses as well as liver, kidney, and cardiac dysfunction may play important roles in determining final outcomes in patients with severe illness due to COVID-19 infection. Our data may aid clinicians in diagnosing severe cases of COVID-19 and determining the most appropriate treatment strategies for infected patients. Given that Traditional Chinese medicine has been shown to improve outcomes in some cases, additional studies are also required to assess the efficacy of such strategies.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Commission of Wuhan Union Hospital of Tongji Medical College, Huazhong University of Science and Technology. Written informed consent was waived for the emergency of this infectious disease.

AUTHOR CONTRIBUTIONS

CY and WY designed the study, had full access to all data in the study, and took responsibility for the integrity of data and the accuracy of the data analysis. XF and LM contributed to data collection, literature search, and writing of the manuscript. XF and PL had roles in data analysis and data interpretation. All authors contributed to data acquisition and clinical management, and they reviewed and approved the final version of the manuscript.

ACKNOWLEDGMENTS

We thank all the patients and their families involved in this study as well as numerous civilians working together to fight against SARS-CoV-2.

REFERENCES

1. Zhu N, Zhang D, Wang W, Li X, Yang B, Song J, et al. A novel coronavirus from patients with pneumonia in China, 2019. *N Engl J Med.* (2020) 382:727–33. doi: 10.1056/NEJMoa2001017
2. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet.* (2020) 395:497–506. doi: 10.1016/S0140-6736(20)30183-5
3. Guan WJ, Ni ZY, Hu Y, Liang WH, Ou CQ, He JX, et al. Clinical characteristics of Coronavirus Disease 2019 in China. *N Engl J Med.* (2020) 382:1708–20. doi: 10.1101/2020.02.06.20020974
4. Xu XW, Wu XX, Jiang XG, Xu KJ, Ying LJ, Ma CL, et al. Clinical findings in a group of patients infected with the 2019 novel coronavirus

(SARS-Cov-2) outside of Wuhan, China: retrospective case series. *BMJ*. (2020) 368:m606. doi: 10.1136/bmj.m606
5. Yang X, Yu Y, Xu J, Shu H, Xia Ja, Liu H, et al. Clinical course and outcomes of critically ill patients with SARS-CoV-2 pneumonia in Wuhan, China: a single-centered, retrospective, observational study. *Lancet Resp Med*. (2020) 8:475–81. doi: 10.1016/S2213-2600(20)30079-5
6. Jin X, Lian JS, Hu JH, Gao J, Zheng L, Zhang YM, et al. Epidemiological, clinical and virological characteristics of 74 cases of coronavirus-infected disease (2019). (COVID-19) with gastrointestinal symptoms. *Gut*. (2020) 69:1002–100. doi: 10.1136/gutjnl-2020-320926
7. Wu C, Chen X, Cai Y, Xia J, Zhou X, Xu S, et al. Risk factors associated with acute respiratory distress syndrome and death in patients with Coronavirus Disease (2019). pneumonia in Wuhan, China. *JAMA Intern Med*. (2020) 180:1–11. doi: 10.1001/jamainternmed.2020.0994
8. Guo W, Li M, Dong Y, Zhou H, Zhang Z, Tian C, et al. Diabetes is a risk factor for the progression and prognosis of COVID-19. *Diabetes Metab Res Rev*. (2020) doi: 10.1002/dmrr.3319. [Epub ahead of print].
9. Released by National Health Commission National Administration of Traditional Chinese Medicine on March 3. Diagnosis and treatment protocol for novel coronavirus pneumonia (Trial Version 7). *Chin Med J*. (2020) 133:1087–95. doi: 10.1097/CM9.0000000000000819
10. Wu Z, McGoogan J. Characteristics of and important lessons from the Coronavirus Disease 2019. (COVID-19) outbreak in China. *JAMA*. (2020) 323:1239–42. doi: 10.1001/jama.2020.2648
11. Dong Y, Mo X, Hu Y, Qi X, Jiang F, Jiang Z, et al. Epidemiology of COVID-19 among children in China. *Pediatrics*. (2020) 145:e20200702. doi: 10.1542/peds.2020-0702
12. Feng Y, Ling Y, Bai T, Xie Y, Huang J, Li J, et al. COVID-19 with different severity_ a multi-center study of clinical features. *Am J Respir Crit Care Med*. (2020) 201:1380–8. doi: 10.1164/rccm.202002-0445OC
13. Chen N, Zhou M, Dong X, Qu J, Gong F, Han Y, et al. Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study. *Lancet*. (2020) 395:507–13. doi: 10.1016/S0140-6736(20)30211-7
14. Mao L, Jin H, Wang M, Hu Y, Chen S, He Q, et al. Neurologic manifestations of hospitalized patients with Coronavirus Disease 2019 in Wuhan, China. *JAMA Neurol*. (2020) 77:1–9. doi: 10.1001/jamaneurol.2020.1127
15. Wang D, Hu B, Hu C, Zhu F, Liu X, Zhang J, et al. Clinical characteristics of 138 hospitalized patients with 2019 novel coronavirus-infected pneumonia in Wuhan, China. *JAMA*. (2020) 323:1061–69. doi: 10.1001/jama.2020.1585
16. Wang Z, Chen X, Lu Y, Chen F, Zhang W. Clinical characteristics and therapeutic procedure for four cases with 2019 novel coronavirus pneumonia receiving combined Chinese and Western medicine treatment. *Biosci Trends*. (2020) 14:64–8. doi: 10.5582/bst.2020.01030
17. Gu J, Gong E, Zhang B, Zheng J, Gao Z, Zhong Y, et al. Multiple organ infection the pathogenesis of SARS. *J Exp Med*. (2005) 202:415–24. doi: 10.1084/jem.20050828
18. Chen T, Wu D, Chen H, Yan W, Yang D, Chen G, et al. Clinical characteristics of 113 deceased patients with coronavirus disease 2019: retrospective study. *BMJ*. (2020) 368:m1091. doi: 10.1136/bmj.m1091
19. Tan L, Wang Q, Zhang D, Ding J, Huang Q, Tang YQ, et al. Lymphopenia predicts disease severity of COVID-19: a descriptive and predictive study. *Signal Transduct Target Ther*. (2020) 5:33. doi: 10.1038/s41392-020-0148-4
20. Chen G, Wu D, Guo W, Cao Y, Huang D, Wang H, et al. Clinical and immunological features of severe and moderate coronavirus disease 2019. *J Clin Invest*. (2020) 130:2620–9. doi: 10.1101/2020.02.16.20023903
21. Emanueli C, Badimon L, Martelli F, Potocnjak I, Carpusca I, Robinson EL, et al. Call to action for the cardiovascular side of COVID-19. *Eur Heart J*. (2020) 41:1796–7. doi: 10.1093/eurheartj/ehaa301
22. Qi X, Liu C, Jiang Z, Gu Y, Zhang G, Shao C, et al. Multicenter analysis of clinical characteristics and outcome of COVID-19 patients with liver injury. *J Hepatol*. (2020) 73:45–8. doi: 10.1016/j.jhep.2020.04.010
23. Cai Q, Huang D, Yu H, Zhu Z, Xia Z, Su Y, et al. Characteristics of liver tests in COVID-19 patients. *J Hepatol*. (2020) doi: 10.1016/j.jhep.2020.04.006. [Epub ahead of print].
24. Ren JL, Zhang AH, Wang XJ. Traditional Chinese medicine for COVID-19 treatment. *Pharmacol Res*. (2020) 155:104743. doi: 10.1016/j.phrs.2020.104743
25. Yang Y, Islam MS, Wang J, Li Y, Chen X. Traditional Chinese medicine in the treatment of patients infected with 2019-new Coronavirus (SARS-CoV-2): a review and perspective. *Int J Biol Sci*. (2020) 16:1708–17. doi: 10.7150/ijbs.45538
26. Wan S, Yi Q, Fan S, Lv J, Zhang X, Guo L, et al. Characteristics of lymphocyte subsets and cytokines in peripheral blood of 123 hospitalized patients with 2019 novel coronavirus pneumonia (NCP). *medRxiv*. (2020) doi: 10.1101/2020.02.10.20021832. [Epub ahead of print].
27. Ye Q, Wang B, Mao J. Cytokine storm in COVID-19 and treatment. *J Infect*. (2020) 80:607–13. doi: 10.1016/j.jinf.2020.03.037
28. Chen L, Liu HG, Liu W, Liu J, Liu K, Shang J, et al. Analysis of clinical features of 29 patients with 2019 novel coronavirus pneumonia. *Zhonghua Jie He He Hu Xi Za Zhi*. (2020) 43:E005. doi: 10.3760/cma.j.issn.1001-0939.2020.0005
29. Mehta P, McAuley DF, Brown M, Sanchez E, Tattersall RS, Manson JJ. COVID-19: consider cytokine storm syndromes and immunosuppression. *Lancet*. (2020) 395:1033–4. doi: 10.1016/S0140-6736(20)30628-0
30. Henderson LA, Canna SW, Schulert GS, Volpi S, Lee PY, Kernan KF, et al. On the alert for cytokine storm: Immunopathology in COVID-19. *Arthritis Rheumatol*. (2020) 72:1059–63. doi: 10.1002/art.41285
31. Brigham KL. Lower tidal volume ventilation and plasma cytokine markers of inflammation in patients with acute lung injury. *Curr Infect Dis Rep*. (2005) 7:327–8. doi: 10.1007/s11908-005-0004-2
32. Lew TW, Kwek TK, Tai D, Earnest A, Loo S, Singh K, et al. G. Acute respiratory distress syndrome in critically ill patients with severe acute respiratory syndrome. *JAMA*. (2003) 290:374–80. doi: 10.1001/jama.290.3.374
33. Kim KD, Zhao J, Auh S, Yang X, Du P, Tang H, et al. Adaptive immune cells temper initial innate responses. *Nat Med*. (2007) 13:1248–52. doi: 10.1038/nm1633
34. Zhao J, Zhao J, Perlman S. T cell responses are required for protection from clinical disease and for virus clearance in severe acute respiratory syndrome coronavirus-infected mice. *J Virol*. (2010) 84:9318–25. doi: 10.1128/JVI.01049-10

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pediatrics

CASE REPORT
published: 06 August 2020
doi: 10.3389/fped.2020.00507

Case Report: Benign Infantile Seizures Temporally Associated With COVID-19

Marcos García-Howard[1], Mercedes Herranz-Aguirre[2], Laura Moreno-Galarraga[3,4], María Urretavizcaya-Martínez[1], Josune Alegría-Echauri[1], Nerea Gorría-Redondo[5], Laura Planas-Serra[6,7], Agatha Schlüter[6,7], Marta Gut[8,9], Aurora Pujol[6,7,10] and Sergio Aguilera-Albesa[3,5]**

[1] Department of Pediatrics, CHN, Navarra Health Service Hospital, Pamplona, Spain, [2] Pediatric Infectious Disease Unit, Department of Pediatrics, CHN, Navarra Health Service Hospital, Pamplona, Spain, [3] NavarraBioMed and IdiSNA, Health Research Institute, Pamplona, Spain, [4] Pediatric Respiratory Medicine, Department of Pediatrics, CHN, Navarra Health Service Hospital, Pamplona, Spain, [5] Pediatric Neurology Unit, Department of Pediatrics, CHN, Navarra Health Service Hospital, Pamplona, Spain, [6] Neurometabolic Diseases Laboratory, Bellvitge Biomedical Research Institute (IDIBELL), Barcelona, Spain, [7] Center for Biomedical Research on Rare Diseases (CIBERER), ISCIII, Madrid, Spain, [8] CNAG-CRG, Centre for Genomic Regulation, Barcelona Institute of Science and Technology, Barcelona, Spain, [9] Universitat Pompeu Fabra (UPF), Barcelona, Spain, [10] Catalan Institution of Research and Advanced Studies (ICREA), Barcelona, Spain

OPEN ACCESS

Edited by:
Christoph Aebi,
University Children's Hospital
Bern, Switzerland

Reviewed by:
Federico Vigevano,
Bambino Gesù Children Hospital
(IRCCS), Italy
Rudá Alessi,
Faculdade de Medicina Do
ABC, Brazil

***Correspondence:**
Sergio Aguilera-Albesa
saguilea@navarra.es
Aurora Pujol
apujol@idibell.cat

Specialty section:
This article was submitted to
Pediatric Infectious Diseases,
a section of the journal
Frontiers in Pediatrics

Received: 03 May 2020
Accepted: 17 July 2020
Published: 06 August 2020

Citation:
García-Howard M, Herranz-Aguirre M,
Moreno-Galarraga L,
Urretavizcaya-Martínez M,
Alegría-Echauri J, Gorría-Redondo N,
Planas-Serra L, Schlüter A, Gut M,
Pujol A and Aguilera-Albesa S (2020)
Case Report: Benign Infantile Seizures
Temporally Associated With
COVID-19. Front. Pediatr. 8:507.
doi: 10.3389/fped.2020.00507

Background: Non-febrile illness seizures may present in previously healthy children as afebrile seizures associated with minor infections, such as mild gastroenteritis or respiratory tract infections, and are linked to a genetic predisposition. For the novel human coronavirus SARS-CoV-2, causing COVID-19, fever, cough, and gastrointestinal complaints are the most common symptoms in children, and a hyperimmune response may be present. No detailed temporally associated neurological complications have been documented in pediatric case series so far.

Case description: We present the case of a 3-months-old girl with non-febrile repeated seizures in a COVID-19 family setting. The infant started with a mild fever and cough that lasted for 2 days. At day 6 from onset, the girl presented with two focal motor seizures with impaired consciousness and awareness. All investigations ruled out signs of meningo-encephalitis or active epilepsy, including normal electroencephalogram and cerebral magnetic resonance imaging. PCR from nasal and throat swabs was positive for SARS-CoV-2. Remarkably, blood ferritin and D-dimer levels were increased. At day 9, the infant presented another afebrile motor seizure, and levetiracetam dose was modified there was a favorable response within 3 months of the follow-up. Much interest has been raised with regards to host genetic determinants to disease severity and susceptibility to COVID-19. We thus performed whole exome sequencing, revealing a pathogenic frameshift mutation in the PRRT2 gene in both the mother and the infant. The mother had presented two late infantile febrile convulsions with normal outcome afterwards.

Discussion: The hyperimmune response described in adult cases with COVID-19 can be seen in infants, even in the absence of respiratory symptoms. Moreover, COVID-19 may present in infants as non-febrile seizures, triggering early onset seizures in infants with a genetic predisposition. In this pandemic situation, precision medicine using

massive sequencing can shed light on underlying molecular mechanisms driving the host response to COVID-19.

Keywords: coronavirus, SARS-CoV-2, COVID-19, pediatric COVID-19, non-febrile seizures, afebrile seizures, PRRT2 mutations, benign familial infantile epilepsy

INTRODUCTION

Non-febrile illness seizures are described as afebrile seizures associated with minor infections in previously healthy children. Seizures occur mainly in infants in the setting of acute infections, such as mild gastroenteritis or respiratory tract infections, without structural correlate or hydro-electrolytic imbalance (1–4). Rotaviruses are frequently found in non-febrile convulsions associated with gastroenteritis, and noroviruses have been recently identified as an emergent pathogen in these cases (1, 5). In infants with non-febrile seizures related to respiratory tract infections, common seasonal viruses, such as influenza, respiratory syncytial virus (RSV), and metapneumovirus, have been pointed out as plausible causative pathogens (3, 4, 6).

Human coronavirus (HCoV) causes respiratory infections with a seasonal pattern in children, and in some cases, extra-pulmonar manifestations have been described. It has been increasingly recognized that HCoV shows some neurotropism due to its capacity to reach the central nervous system after the nasal infection, shown for HCoV-OC43 and HCoV-NL63 (7, 8). Neurological complications of common HCoV infections have been reported, including febrile seizures, convulsions, loss of consciousness, encephalomyelitis, and encephalitis (9). Another HCoV, SARS-CoV, emerged in Guandong province, southern China, in 2002, and it spread to many countries and caused severe lower respiratory tract infection with an overall case-fatality rate of 10%. The SARS-CoV was associated with milder disease in children compared to adults, with some case series reporting febrile seizures in 10% of a total sample of 41 children (10–12). Fortunately, no human SARS-CoV infections have been identified since July 2003 (8).

In December 2019, a novel HCoV (SARS-CoV-2) was reported from Wuhan city, Hubei province, China, and it rapidly spread worldwide causing a pandemic outbreak by March 2020, producing a respiratory disease called COVID-19. Initial case series have shown that children present milder clinical symptoms than adults and that most pediatric cases were infected in family clusters. Fever, cough, respiratory distress, myalgia, and gastrointestinal complaints are the most common symptoms (13–18), but no detailed neurological complications have been documented in pediatric case series so far.

In this article, we describe the case of a 3-months-old girl with non-febrile repeated seizures in a COVID-19 family setting. Whole exome sequencing was applied and revealed an underlying genetic pathogenic variant that may cause the clinical presentation.

CASE PRESENTATION

A previously healthy, with uneventful pregnancy and delivery, 3-months-old girl was admitted to the pediatric emergency department early morning on April 1 after her mother reported two episodes of convulsions without fever. During the night, the mother, who was a nurse, reported a first episode of clonic movements of the face with tonic posture of extremities and trismus, without consciousness, lasting 3 min approximately. Few hours later, the infant presented with a second episode, described as staring gaze, clonic movements of the face and right extremities, and repeating sucking movements of the mouth, lasting <5 min. At admission, vital constants were normal; physical and neurological examination showed mild hypotonia and drowsiness without focal deficits. The mother informed us that, on March 27–28, the infant had presented with a low fever of <38.1°C, rhinorrhea, cough, and diarrhea with subsequent improvement. No fever was documented the 3 days before these convulsions. Interestingly, the mother referred herself as having persistent symptoms of anosmia and dysgeusia since March 23, and showed no signs of fever or respiratory symptoms since.

At admission, patient blood tests did not show any abnormalities except for a high ferritin value (385 μg/L; normal values 10–204). PCRs of nasopharyngeal and throat swabs tested positive for SARS-CoV-2. Additional testing for other viruses was negative, including HCoV-NL63, HCoV-OC43, HCoV-229E, RSV, rhinovirus, metapneumovirus, influenza, adenovirus, bocavirus, and enterovirus. Chest x-ray and brain CT scans did not reveal abnormalities; the CSF analysis for cells, glucose, and protein was normal. PCRs for herpes virus family (HSV-1, HSV-2, and VZV) and enterovirus in CSF were negative. Bacteria cultures in blood, urine, and CSF were also negative.

During hospitalization, three interictal electroencephalograms (EEG) and a cerebral 1.5T MRI showed normal results. Levetiracetam was started the 1st day as prophylactic antiepileptic treatment (28 mg/Kg/day), but the infant presented with another afebrile seizure on April 4, consisting of upright tonic eye deviation, clonic movements of face muscles, and tonic posture of four limbs in extension, lasting 90 s. The seizure was recorded by the mother and was checked by pediatric neurologists. Pre-dosing blood levels of levetiracetam were within therapeutic range (15 mcg/mL; normal values 10–40). Hydroxychloroquine was then started on April 4, as compassionate use due to the persistence of seizures in a COVID-19 setting, at a dosage of 6.5 mg/Kg/day for 5 days with excellent tolerance. Blood test controls revealed a sustained decrease of ferritin values, with a late increase

of D-dimer levels. Unfortunately, D-dimer control was not possible due to technical procedures. The rest of the lab tests were within normal limits. Following discharge, the patient was followed as an outpatient, and she presented with normal neurological development and an absence of seizures. A timeline of clinical course, lab test, and investigations is summarized in **Figure 1**. Informed and written consent to publish clinical details was obtained from the parents. Additionally, during hospitalization, the patient and her mother were included in a collaborative study of genomic medicine for identifying genetic variants causing hyperimmunity due to SARS-CoV-2 infection. The local ethics committee approved the study. Whole exome sequencing was performed, and prioritized genes were analyzed. No pathogenic variants of susceptibility genes for hyperimmunity were found, but both the infant and the mother carried a loss-of-function variant in the PRRT2 gene (NM_145239.3), c.649dupC (p.Arg217fs), at the heterozygous state. This frameshift variant has been recurrently described in ClinVar as pathogenic. It is associated with benign familial infantile convulsions (OMIM 605751), but it is also allelic to infantile convulsions and choreoathetosis (OMIM 602066) (19). A revision of maternal family history revealed that the mother could have had two convulsions during late infancy, related to mild infections and fever, with normal development afterwards.

DISCUSSION

SARS-CoV-2 infection in children is being increasingly recognized. However, detailed clinical data are still lacking and individual cases, such as the one presented, can shed some light to comprehend the complex systemic manifestations of this disease in the youngest.

A review of the Chinese Center for Disease Control and Prevention on February 24 has shown than <1% of the COVID-19 cases were in children younger than 10 years of age (20). In Wuhan Children's Hospital, China, 1,391 children were tested through February, and a total of 171 (12.3%) were positive for SARS-CoV-2 infection, with a median age of 6.7 years. Of these, 65% presented pneumonia and three required invasive mechanical ventilation. Only 18% of the positive children were infants younger than 1 year of age, and no cases were reported with neurological features. In Spain, by March 16, 41 of the 4,695 confirmed cases (0.8%) in the Madrid region were children younger than 18 years, and 60% of the pediatric cases required hospitalization (21). In a systematic review of SARS-CoV-2 infection in children, Castagnoli et al. found, by April 22, 444 participants younger than 10 years of age, but no details about clinical symptoms were revealed (22).

Our infant presented with afebrile seizures during the course of COVID-19, some of them with focal semiology, and these were

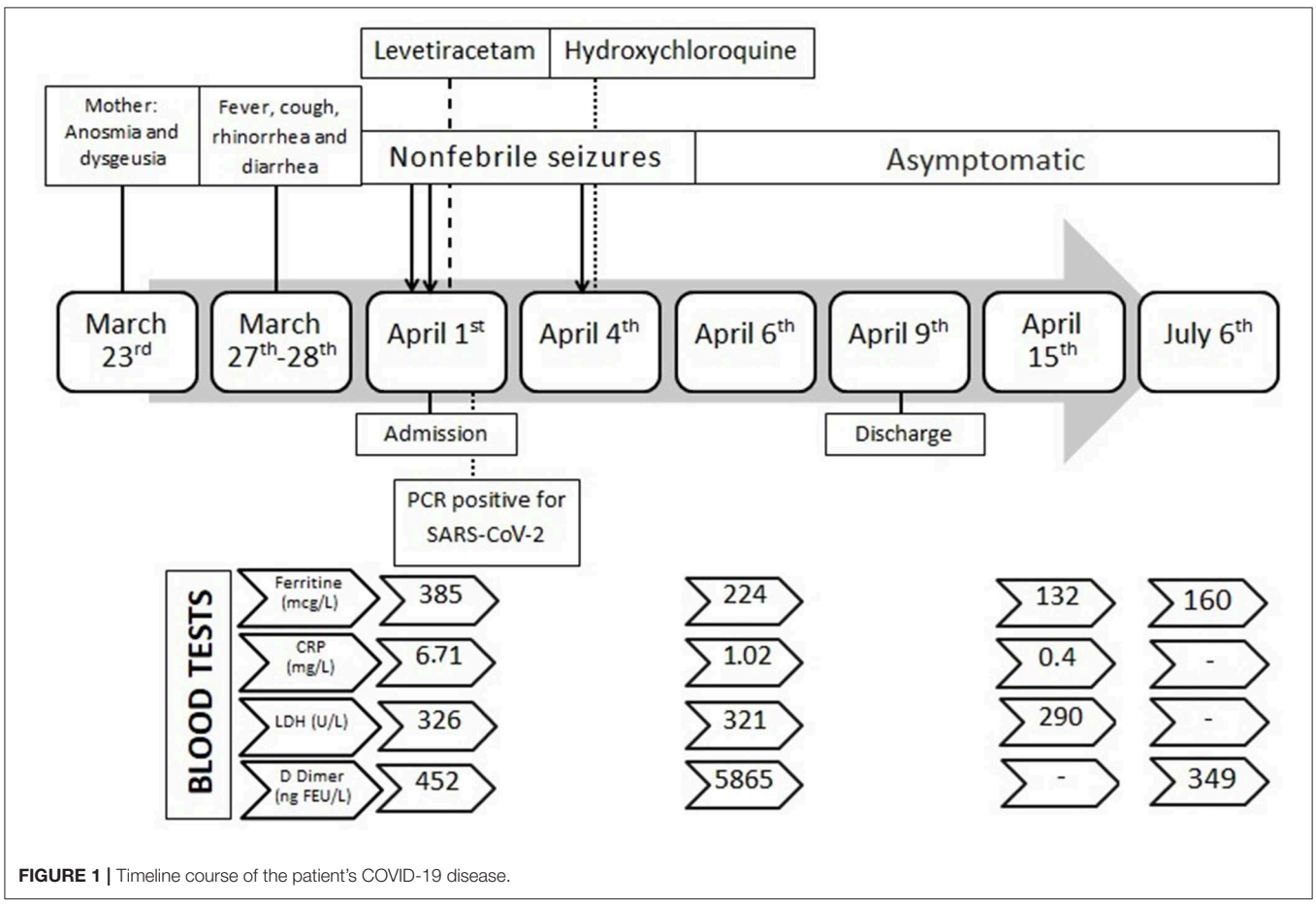


FIGURE 1 | Timeline course of the patient's COVID-19 disease.

not associated with signs of encephalitis, structural damage, or other concomitant infection. The clinical picture is compatible with the definition of non-febrile illness seizures, which occur in association with an acute infection the week before or 3 days after the seizure, although without presenting with fever on the day of the seizure (3, 23).

Besides respiratory symptoms, HCoV infections may present with febrile seizures in susceptible infants (9–12). Very recently, a febrile convulsion in a 2-years-old girl with COVID-19 was reported (15). However, several studies have suggested that non-febrile illness seizures are a different seizure category from febrile seizures or unprovoked seizures. Non-febrile illness seizures may share some genetic predisposition in a similar manner as febrile seizures or epilepsy, and, as with febrile seizures, the prognosis is favorable in most cases (3).

This pandemic has sparked an interest in genomic medicine to elucidate host determinants of phenotype severity (24, 25). We thus performed whole exome sequencing as described (26) and uncovered the genetic predisposition of the infant to develop afebrile seizures due to a well-known recurrent pathogenic PRRT2 mutation associated with benign familial infantile convulsions (OMIM 605751) and infantile convulsions and choreoathetosis (OMIM 602066) (19). In the mother, the phenotype is benign and self-limited, without movement disorders. Nonetheless, a long-term follow-up is required to detect the possible development of dyskinesias in the infant.

Regarding other investigations in this case, ferritin levels were increased at admission, on day 6 since symptoms onset, and they progressively decreased over time. D-dimers were within normal limits at admission but increased during hospitalization. These findings resemble the hyperimmune response found is adult COVID-19 severe cases, a major driver of adverse outcome (27, 28). Same findings are being reported in severe COVID-19 in children (8), and, during the SARS-CoV epidemic of 2002, some patients presented with decreased lymphocyte count and increased levels of LDH and D-dimers.

Therapeutic strategies and evidence-based protocols for COVID-19 treatment in children are still lacking. On April 4, we thus decided to treat our infant with hydroxychloroquine since this drug is renowned for its antiviral and immunomodulating properties (29), and it has been previously used in young infants (30–33). Preliminary results of hydroxychloroquine on adults COVID-19 clinical trials suggested that 600 mg daily may decrease viral load in nasal swabs (34), but further studies has raised concerns about its use during hospitalization (35). On July 4, the WHO International Steering Committee discontinued clinical trials for hydroxychloroquine in hospitalized patients (www.who.int/news-room).

One limitation of this case is related to the storing of the biological samples. PCR of SARS-CoV-2 in CSF was not available at that moment, and the sample was not stored. Moreover, hyperimmune response in younger children is an uncommon phenomenon and warrants further research in samples of the patients. We would like to encourage pediatricians to collect and store biological samples of patients with COVID-19 for further analysis, as it would be useful for future research.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Complejo Hospitalario de Navarra. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the minor(s)' legal guardian/next of kin for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

SA-A and MH-A conceived and designed the manuscript. MG-H wrote the first draft. LM-G, MU-M, JA-E, and NG-R provided data acquisition, clinical details of the patient, and reviewed the literature. LP-S, AS, and MG generated and analyzed the exomes using the CNAG pipeline. AP provided funding and interpreted the variants in its clinical context. SA-A wrote the final manuscript. AP and SA-A added and refined discussion and final conclusions. All authors reviewed and approved the final version as submitted, and agreed to be accountable for all aspects of the work.

FUNDING

Whole exome sequencing was carried out thanks to the EASIGenomics COVID-19/ PID1234219 grant to AP and the Illumina Match Funds covid-19 initiative. Pediatric epilepsy studies in our hospital are carried out thanks to Fundación Alicia Koplowitz grant to SA-A.

ACKNOWLEDGMENTS

The authors would like to thank the patient's family for their cooperation and for consenting to the publication of this manuscript. They will also like to thank the enormous effort done by all our hospital and Spanish health workers in the fight against the current COVID-19 pandemic.

REFERENCES

1. Uemura N, Okumura A, Negoro T, Watanabe K. Clinical features of benign convulsions with mild gastroenteritis. *Brain Dev*. (2002) 24:745–9. doi: 10.1016/S0387-7604(02)00097-9

2. Durá-Travé T, Yoldi-Petri ME, Molins-Castiella T, Souto-Hernández S, Aguilera-Albesa S. Infantile convulsions with mild gastroenteritis: epidemiological and clinical characteristics and outcome. *Rev Neurol*. (2010) 5:12–8. doi: 10.33588/rn.5101.2010018

3. Zhang T, Ma J, Gan X, Xiao N. Are afebrile seizures associated with minor infections a single seizure category? A hospital-based prospective cohort study on outcomes of first afebrile seizure in early childhood. *Epilepsia*. (2014) 55:1001–8. doi: 10.1111/epi.12651
4. Conejo Moreno D, Rodríguez Fernández C, Ruíz Ayúcar de la Vega I, Ortiz Madinaveitia S, Hedrera Fernández A, Maldonado Ruiz. E, et al. Para-infectious seizures: a retrospective multicentre study. *An Pediatr*. (2016) 85:300–4. doi: 10.1016/j.anpede.2016.01.012
5. Ma X, Luan S, Zhao Y, Lv X, Zhang R. Clinical characteristics and follow-up of benign convulsions with mild gastroenteritis among children. *Medicine*. (2019) 98:e14082. doi: 10.1097/MD.0000000000014082
6. Cha T, Choi YJ, Oh JW, Kim CR, Park DW, Seol IJ, et al. Respiratory syncytial virus-associated seizures in Korean children, 2011–2016. *Korean J Pediatr*. (2019) 62:131–7. doi: 10.3345/kjp.2018.07066
7. St-Jean JR, Jacomy H, Desforges M, Vabret A, Freymuth F, Talbot PJ. Human respiratory coronavirus OC43: genetic stability and neuroinvasion. *J Virol*. (2004) 78:8824–34. doi: 10.1128/JVI.78.16.8824-8834.2004
8. Zimmermann P, Curtis N. Coronavirus infections in children including COVID-19: an overview of the epidemiology, clinical features, diagnosis, treatment and prevention options in children. *Pediatr Infect Dis J*. (2020) 39:355–68. doi: 10.1097/INF.0000000000002660
9. Bohmwald K, Gálvez NMS, Ríos M, Kalergis AM. Neurologic alterations due to respiratory virus infections. *Front Cell Neurosci*. (2018) 12:386. doi: 10.3389/fncel.2018.00386
10. Hon KL, Leung CW, Cheng WT, Chan PK, Chu WC, Kwan YW, et al. Clinical presentations and outcome of severe acute respiratory syndrome in children. *Lancet*. (2003) 361:1701–3. doi: 10.1016/S0140-6736(03)13364-8
11. Chiu WK, Cheung PC, Ng KL, Ip PL, Sugunan VK, Luk DC, et al. Severe acute respiratory syndrome in children: experience in a regional hospital in Hong Kong. *Pediatr Crit Care Med*. (2003) 4:279–83. doi: 10.1097/01.PCC.0000077079.42302.81
12. Bitnun A, Allen U, Heurter H, King SM, Opavsky MA, Ford-Jones EL, et al. Children hospitalized with severe acute respiratory syndrome-related illness in Toronto. *Pediatrics*. (2003) 112:e261. doi: 10.1542/peds.112.4.e261
13. Chan JF, Yuan S, Kok KH, To KK, Chu H, Yang J, et al. A familial cluster of pneumonia associated with the 2019 novel coronavirus indicating person-to-person transmission: a study of a family cluster. *Lancet*. (2020) 395:514–23. doi: 10.1016/S0140-6736(20)30154-9
14. Zhu N, Zhang D, Wang W, Li X, Yang B, Song J, et al. A novel coronavirus from patients with pneumonia in China, 2019. *N Engl J Med*. (2020) 382:727–33. doi: 10.1056/NEJMoa2001017
15. Tan YP, Tan BY, Pan J, Wu J, Zeng SZ, Wei HY. Epidemiologic and clinical characteristics of 10 children with coronavirus disease 2019 in Changsha, China. *J Clin Virol*. (2020) 127:104353. doi: 10.1016/j.jcv.2020.104353
16. Shen KL, Yang YH, Jiang RM, Wang TY, Zhao DC, Jiang Y, et al. Updated diagnosis, treatment and prevention of COVID-19 in children: experts' consensus statement (condensed version of the second edition). *World J Pediatr*. (2020) 16:232–9. doi: 10.1007/s12519-020-00362-4
17. Dong Y, Mo X, Hu Y, Qi X, Jiang F, Jiang Z, et al. Epidemiological characteristics of 2143 pediatric patients with 2019 coronavirus disease in China. *Pediatrics*. (2020) 58:712–3. doi: 10.1016/j.jemermed.2020.04.006
18. CDC COVID-19 response team. Coronavirus disease 2019 in children—United States, February 12-April 2, 2020. *MMWR Morb Mortal Wkly Rep*. (2020) 69:422–6. doi: 10.15585/mmwr.mm6914e4
19. Heron SE, Grinton BE, Kivity S, Afawi Z, Zuberi SM, Hughes JN, et al. PRRT2 mutations cause benign familial infantile epilepsy and infantile convulsions with choreoathetosis syndrome. *Am J Hum Genet*. (2012) 90:152–60. doi: 10.1016/j.ajhg.2011.12.003
20. Wu Z, McGoogan JM. Characteristics of and important lessons from the coronavirus disease 2019 (COVID-19) outbreak in China: summary of a report of 72 314 cases from the Chinese Center for Disease Control and Prevention. *JAMA*. (2020). doi: 10.1001/jama.2020.2648. [Epub ahead of print].
21. Tagarro A, Epalza C, Santos M, Sanz-Santaeufemia FJ, Otheo E, Moraleda C, et al. Screening and severity of coronavirus disease 2019 (COVID-19) in children in Madrid, Spain. *JAMA Pediatr*. (2020) e201346. doi: 10.1001/jamapediatrics.2020.1346. [Epub ahead of print].
22. Castagnoli R, Votto M, Licari A, Brambilla I, Bruno R, Perlini S, et al. Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) infection in children and adolescents. A systematic review. *JAMA Pediatr*. (2020). doi: 10.1001/jamapediatrics.2020.1467. [Epub ahead of print].
23. Zerr DM, Blume HK, Berg AT, Del Beccaro MA, Gospe SM Jr, Allpress AL, et al. Non-febrile illness seizures: a unique seizure category? *Epilepsia*. (2005) 46:952–5. doi: 10.1111/j.1528-1167.2005.65204.x
24. Casanova JL, Su HC. A global effort to define the human genetics of protective immunity to SARS-CoV-2 infection. *Cell*. (2020) 181:1194–1199. doi: 10.1016/j.cell.2020.05.016
25. The COVID-19 Host Genetics Initiative. The COVID-19 Host Genetics Initiative, a global initiative to elucidate the role of host genetic factors in susceptibility and severity of the SARS-CoV-2 virus pandemic. *Eur J Human Genet*. (2020) 28:715–8. doi: 10.1038/s41431-020-0636-6
26. Pant DC, Dorboz I, Schluter A, Fourcade S, Launay N, Joya J, et al. Loss of the sphingolipid desaturase DEGS1 causes hypomyelinating leukodystrophy. *J Clin Invest*. (2019) 129:1240–56. doi: 10.1172/JCI123959
27. Qin C, Zhou L, Hu Z, Zhang S, Yang S, Tao Y, et al. Dysregulation of immune response in patients with COVID-19 in Wuhan, China. *Clin Infect Dis*. (2020) ciaa248. doi: 10.2139/ssrn.3541136. [Epub ahead of print].
28. Mehta P, McAuley DF, Brown M, Sanchez E, Tattersall RS, Manson JJ, et al. COVID-19: consider cytokine storm syndromes and immunosuppression. *Lancet*. (2020) 395:1033–4. doi: 10.1016/S0140-6736(20)30628-0
29. Sturrock BR, Chevassut TJ. Chloroquine and COVID-19—a potential game changer? *Clin Med*. (2020) 20:278–1. doi: 10.7861/clinmed.2020-0129
30. White NJ. The treatment of malaria. *N Engl J Med*. (1996) 335:800–6. doi: 10.1056/NEJM199609123351107
31. Colson P, Rolain JM, Raoult D. Chloroquine for the 2019 novel coronavirus SARS-CoV-2. *Int J Antimicrob Agents*. (2020) 55:105923. doi: 10.1016/j.ijantimicag.2020.105923
32. Vincent MJ, Bergeron E, Benjannet S, Erickson BR, Rollin PE, Ksiazek TG, et al. Chloroquine is a potent inhibitor of SARS coronavirus infection and spread. *Virol J*. (2005) 2:69. doi: 10.1186/1743-422X-2-69
33. Yao X, Ye F, Zhang M, Cui C, Huang B, Niu P, et al. *In vitro* antiviral activity and projection of optimized dosing design of hydroxychloroquine for the treatment of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). *Clin Infect Dis*. (2020) ciaa237. doi: 10.1093/cid/ciaa237. [Epub ahead of print].
34. Gautret P, Lagier JC, Parola P, Hoang VT, Meddeb L, Mailhe M, et al. Hydroxychloroquine and azithromycin as a treatment of COVID-19: results of an open-label non-randomized clinical trial. *Int J Antimicrob Agents*. (2020) 105949. doi: 10.1016/j.ijantimicag.2020.105949. [Epub ahead of print].
35. Alexander PE, Debono VB, Mammen MJ, Iorio A, Aryal K, Berg D, et al. COVID-19 coronavirus research has overall low methodological quality thus far: case in point for chloroquine/hydroxychloroquine. *J Clin Epidemiol*. (2020) 123:120–6. doi: 10.1016/j.jclinepi.2020.04.016

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Public Health

OPINION
published: 06 August 2020
doi: 10.3389/fpubh.2020.00437

Convalescent Plasma: A Potential Life-Saving Therapy for Coronavirus Disease 2019 (COVID-19)

Ahmed N. Alghamdi[1] *and Ahmed S. Abdel-Moneim*[1,2]*

[1] *Microbiology Department, College of Medicine, Taif University, Al-Taif, Saudi Arabia,* [2] *Virology Department, Faculty of Veterinary Medicine, Beni-Suef University, Beni Suef, Egypt*

Keywords: COVID-19, SARS-CoV-2, coronavirus, plasma therapy, immunetherapy

INTRODUCTION

The last two decades witnessed the emergence of three zoonotic coronaviruses that crossed the species barrier and caused outbreaks in humans: severe acute respiratory syndrome coronavirus (SARS-CoV) in 2002, Middle East respiratory syndrome coronavirus (MERS-CoV) in 2012, and most recently, severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) in 2019 (1). All three viruses are β-coronaviruses belonging to the *Orthocoronavirinae* subfamily in the *Coronaviriade* viral family (1). Similar to SARS-CoV, the newly emerged SARS-CoV-2 utilizes the angiotensin-converting enzyme 2 (ACE2) cellular receptor for cell entry (2). Although many drugs have been proposed as potential therapeutic agents for coronavirus disease 2019 (COVID-19), no specific antiviral agent has been proven effective to date (3). Convalescent plasma is effective in treating many viral conditions, including respiratory infections (4). Hence, the therapeutic potential of convalescent plasma for COVID-19 is a noteworthy topic. This viewpoint discusses the plausibility of using convalescent plasma from COVID-19 recovered patients as an effective, feasible therapeutic intervention for COVID-19.

HISTORY

Convalescent plasma has been used since the 1890s to treat several infectious diseases (5). In the early 20th century, convalescent sera obtained from recovered individuals during outbreaks of viral etiology, such as influenza and measles, were used for therapeutic purposes (5). In the early 21st century, convalescent plasma was utilized to increase the survival rate among critically ill patients during the H1N1 pandemic in 2009, as well as during the SARS-CoV and MERS-CoV outbreaks in 2002 and 2012, respectively (5). Thirty-two studies demonstrated a consistent reduction in mortality among convalescent plasma-treated patients with severe SARS and influenza infections without convalescent plasma-related adverse effects (6). The pooled data from 27 out of these 32 studies revealed a statistically significant reduction in the pooled odds of death among the convalescent plasma-treated group compared with the control group (6). Further, during the Ebola virus epidemic in West Africa in 2014, convalescent plasma therapy (CPT) was used empirically, as recommended by the World Health Organization (7).

MODE OF ACTION

Convalescent plasma exemplifies passive immune therapy, which combats invading pathogens by administering antibodies (2). It is hypothesized that polyclonal antibodies in convalescent plasma neutralize the circulating initial inoculum of microbes and facilitate antibody-dependent cellular cytotoxicity and phagocytosis (5). In the context of CPT for COVID-19, the antibodies are anticipated to employ both immune mechanisms, but the main one would be neutralization, which occurs when neutralizing antibodies block SARS-CoV-2 spike proteins,

OPEN ACCESS

Edited by:
Alexander Rodriguez-Palacios, Case Western Reserve University, United States

Reviewed by:
Rima Abdallah Moghnieh, Makassed General Hospital, Lebanon
Giuseppe Di Lorenzo, Azienda Sanitaria Locale Salerno, Italy

***Correspondence:**
Ahmed S. Abdel-Moneim
asa@tu.edu.sa;
asa@bsu.edu.eg

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Public Health

Received: *16 April 2020*
Accepted: *16 July 2020*
Published: *06 August 2020*

Citation:
Alghamdi AN and Abdel-Moneim AS (2020) Convalescent Plasma: A Potential Life-Saving Therapy for Coronavirus Disease 2019 (COVID-19). Front. Public Health 8:437. doi: 10.3389/fpubh.2020.00437

thereby aborting viral entry (2, 4, 5). Hosts naturally develop antibodies 10–14 days post-infection; therefore, convalescent plasma administration before seroconversion is believed to be more therapeutically effective (4, 5).

CPT IN TREATING EMERGING CORONAVIRUSES

CPT for SARS and MERS

Several studies have shown a favorable outcome of CPT in treating infections caused by emerging coronaviruses (3, 5, 7–10). A retrospective non-randomized comparison study addressed the outcome of 40 severely affected SARS-CoV patients, where the treatment group ($n = 19$) received SARS convalescent plasma while the control group was kept only on methylprednisolone after both groups had finished an empirical combination of ribavirin and methylprednisolone (8). Patients in the treatment group were given 200–400 ml of convalescent plasma obtained using an apheresis device from SARS-recovered individuals with a SARS-IgG titer ranging from 160 to 2,560 (8). In 2003, researchers addressed the outcome in 80 SARS-CoV-infected patients on convalescent plasma (7). The outcome was deemed to be good if the hospital admission lasted <23 days post-onset. The SARS-CoV convalescent plasma volume used in this study ranged from 160 to 640 ml with a SARS-IgG titer ranging from 160 to 2,560 (7). The mortality rate among those 80 critical SARS patients was 12.5%, while the overall mortality rate when the SARS epidemic struck Hong Kong in 2003 was 17% (8). Moreover, this study found that convalescent plasma administration before the 14th day post-onset is associated with a better outcome than its administration after this point (58.3 vs. 15.6%). It was also evident from this study that convalescent plasma administration in SARS-CoV PCR-positive but SARS-seronegative patients was more therapeutically effective than in SARS-CoV PCR-positive and SARS-seropositive patients (66.7 vs. 20%) (7). These findings are in accordance with the notion that the effectiveness of CPT is directly related to its early administration prior to seroconversion (3, 5, 8). A study involving three critical SARS-CoV-infected patients who were treated with 500 ml SARS-CoV convalescent plasma of SARS-CoV IgG titer >640 infusion each showed clinical improvement followed by viral burden decline (9). CPT was used to treat three critically ill MERS cases in South Korea (10). The study concluded that effective convalescent plasma treatment was associated with a MERS-IgG titer ≥80, while an IgG titer of 40 was ineffective (10). Further, the neutralization activity could be predicted by ELISA-IgG without conducting sophisticated BSL-3 laboratory-dependent procedures, such as the plaque reduction neutralization test. At a cutoff optical density of 1.6 and 1.9, the specificity of ELISA-IgG in predicting the neutralization activity was ≥95 and 100%, respectively (10).

CPT for COVID-19

In an uncontrolled case series, five ARDS-complicated COVID-19 patients were on mechanical ventilation, four of whom were ≥50 years of age. They received 400 ml of convalescent plasma infusion each immediately after being obtained by apheresis from ABO-compatible donors (3). The convalescent plasma had an IgG titer >1000 and a neutralization titer >40. Following plasma transfusion, all patients manifested a restored normal body temperature within 3 days and the range of their PaO_2/FiO_2 improved from 172–276 to 284–366 within 12 days (3) (**Table 1**). Further, viral load and inflammatory cytokines started to decline while serological responses began to mount after the CPT. Moreover, three out of the five patients were extubated and discharged (3). Anecdotal pieces of evidence on the safety and efficacy of convalescent plasma in treating COVID-19 have been reported (11). After infusion with 200 ml volume and ≥640 neutralization titer convalescent plasma, 10 critical COVID-19 patients on supportive and antiviral treatments improved in terms of clinical and laboratory parameters (11). Post-plasma infusion, fever, and respiratory symptoms subsided within 3 days while RNAemia took 6 days to become undetected. No serious adverse reactions were reported (11) (**Table 1**). Another four severe SARS-CoV-2-infected cases on supportive and antiviral therapy improved following convalescent plasma administration with no adverse effects. The volume of CPT ranged from 200 to 2,400 ml (12) (**Table 1**). The first reported use of CPT for COVID-19 in South Korea was on two ARDS-complicated SARS-CoV-2-infected patients (13). Convalescent plasma was obtained from two fully recovered SARS-CoV-2-causing pneumonia cases with anti-SARS-CoV-2 ELISA optical density of 0.586 and 0.532 (cutoff: 0.22). Despite being on lopinavir/ritonavir and hydroxychloroquine, both CPT recipients had been suffering from a worsening course of ARDS-complicated SARS-CoV-2 infection (PaO_2/FiO_2: <100) prior to the convalescent plasma infusion. After 500 ml of convalescent plasma infusion each, both critically ill COVID-19 patients improved in terms of symptoms and infection-related markers, with no adverse effects reported. Their escalating viral loads prior to the plasma administration started to dramatically fall the next day after the convalescent plasma infusion, while their oxygen demand gradually decreased until they were successfully extubated. Although the subjects in this study received methylprednisolone within 2 days prior to convalescent plasma infusion, their viral burden decreased afterwards, suggesting the successful neutralization effect of the administered plasma (13) (**Table 1**). A recent study in Wuhan, China, described the efficacy and safety of CPT on six COVID-19 cases (14). At least 200 ml of convalescent plasma infusion was administered to six laboratory-confirmed COVID-19 cases, five of whom had lower respiratory tract involvement. All convalescent plasma recipients showed clinical improvement without any adverse effects. Yeh et al. linked CPT in COVID-19 to radiological and serological improvements in terms of resolution of COVID-19-related abnormal radiological findings and mounting numbers of anti-SARS-CoV-2 antibodies, respectively (**Table 1**). There was evidence of the clinical benefits of convalescent plasma in those running a late course of COVID-19 even after seroconversion exists (14). Hence, the efficacy of convalescent plasma in relation to seroconversion should be rigorously evaluated. CPT succeeded in lowering the SARS-CoV-2 viral burden, although it was administered after steroids (3, 13), contradicting the general notion that steroids have a

TABLE 1 | Safety and efficacy of convalescent plasma therapy for COVID-19 infected patients.

Study	Participants	Pre-CPT status	CPT	Post-CPT outcome	Adverse Effects	References
1	n:5, 3♂:2♀, age (36–65 y.), HTN & MR (n:1)	ARDS-complicated COVID-19 (n:5), MV (n:5), ECMO (n:1), PaO_2/FiO_2 (range, 172–276), neutralizing Ab (range, 40–160)	Volume (400 ml), neutralizing Ab titer (range, 80–480), administration day (range, 10–22 d. post-admission)	Fever subsided within 3 d. post-CPT (n:5), viral load decline (undetectable within 12 d. post-CPT) (n:5), PaO_2/FiO_2 improved within 12 d. post-CPT (range, 284–366) (n:5), neutralizing Ab titer increased (range, 80–320 and 160–480 at 1st and 3rd d post-CPT, respectively), radiological improvements noticed from the 3rd d post-CPT (n:5), MV removal within 12 d. post-CPT (n:3), ECMO removal within 5 d. post-CPT (n:1), patient discharge (n:3)	None (n:5)	(3)
2	n:10, 6♂:4♀, age (34–78 y.), HTN (n:3), cardiac and cerebrovascular diseases (n:1)	Severe COVID-19 (n:10), MV (n:3), high-flow O_2 (n:3), low-flow O_2 (n:2), SaO_2% (median,93; range, 89–96.5), neutralizing Ab titer (range, 160–640)*	Volume (200 ml), neutralizing Ab titer (≥640), administration day (range, 10–20 d. post-onset)	Symptoms and SaO_2% (median,96; range, 95–96.5) both improved within 3 d post-CPT (n:10), viral load decline (undetectable within 6 d. post-CPT) ‡ (n:7), neutralizing Ab titer increased to 640*† (n:5), radiological improvements noticed within 3 d post-CPT (n:10), MV removal (n:2), high-flow O_2 not needed anymore (n:2), patient discharge (n:3), patient improved and ready for discharge (n:7)	Evanescent facial red spots (n:1), none (n:9)	(11)
3	n:4, 2♂:2♀, Age (31–73 y.), COPD (n:1), HTN (n:1), HTN and CKD (n:1), pregnant (n:1)	ARDS-complicated COVID-19 (n:4), MV (n:3), non-invasive ventilation and high-flow O_2 (n:1), ECMO (n:1)	Volume (range, 200–2,400 ml), administration day (range, 12–19 d post-admission)	Clinical and radiological improvement (n:4), viral load decline within 30 d. post-CPT§ (qRT-PCR undetectability range, 6–30 d. post-CPT) (n:4), MV removal within 20 d. post-CPT§ (n:2), ECMO removal within 7 days post-CPT§ (n:1), Patient discharge (n:3)	None (n:4)	(12)
4	n:2, 1♂:1♀, Age (67 and 71 y.), HTN (n:1)	ARDS-complicated COVID-19, MV (n:2), PaO_2/FiO_2: 86 and 76	Volume: 500 ml, Anti-SARS-2 IgG ELISA: 0.586 and 0.532 (Cut-off: 0.22), administration day: 6th and 10th d post-admission	Clinical and radiological improvement (n:2), viral load decline (undetectable in both patients after 16 and 14 d post-CPT, respectively), PaO_2/FiO_2 in both patients increased to 300 and 230 within 8 and 6 d post-CPT, respectively, MV removal (n:2), patient discharge (n:1)	None (n:2)	(13)
5	n:6, 3♂:3♀, Age (range, 28–75 y.), Sjören syndrome (n:1)	COVID-19 (n:6), clinical and radiological picture of SARS-CoV-2 causing LRTIΩ (n:5)	Volume (range, 200–600 ml), administration day (33–50 d. post-onset)	Clinical and radiological improvementΩ (n:5), viral load decline and eventually undetectable* (n:5), patient discharge* (n:5)	None (n:6)	(14)

HTN, Hypertension; MR, Mitral regurgitation; ARDS, Acute respiratory distress syndrome; MV, Mechanical ventilation; ECMO, Extracorporeal membrane oxygenation; IFN-α, Interferon-alpha; COPD, Chronic obstructive pulmonary disease; CKD, Chronic kidney disease; LRTI, Lower respiratory tract infection.

**One patient's data was unavailable.*

†*Four patients' neutralizing Ab titer remained the same as before the CPT at 640.*

‡*Three patients had already had undetectable SARS-CoV-2 prior to the CPT.*

§*From the first or the only CPT dose received.*

Ω*One patient was SARS-CoV-2 positive and asymptomatic during the CPT after being previously symptomatic with no LRTI involvement.*

counteractive effect on CPT (11). A thorough assessment of the relationship between steroids and CPT may lead to a more effective therapeutic combination. As of this writing, more than 80 clinical trials are aiming to investigate the safety and efficacy of CPT in COVID-19 subjects; however, results have yet to be posted (15). The scarcity of convalescent plasma donors should not be a problem, with millions of fully recovered COVID-19 cases all over the globe.

DRAWBACKS OF USING CPT

The drawbacks associated with CPT include adverse effects, such as transfusion transmissible infections (TTIs) and transfusion-related acute lung injury (TRALI) (2, 5). In addition, its effectivity depends on the neutralization titer (2, 10). However, based on available studies, plasma infusion is a safe medical practice, mainly due to advances in blood banking and transfusion, including ABO compatibility checking and TTI screening and monitoring during and after transfusion (2, 5). Further, in a reported case of TRALI following MERS convalescent plasma infusion, neither anti-human leukocyte antigen nor anti-human neutrophil antigen, both of which are TRALI pathophysiology key players, were detected in the donated plasma (2, 16). Additionally, neutralization activity could be predicted by ELISA-IgG (10).

CONCLUSION

Currently, no specific antiviral agent has been proven for SARS-CoV-2 infection. However, based on available data, it is plausible to consider CPT as an effective, safe, and feasible therapeutic option for COVID-19. Determining the effective dose of convalescent plasma infusion is essential, along with other variables such as the neutralization titer.

AUTHOR CONTRIBUTIONS

ASA contributed conception of the study and critically revised the manuscript. ANA wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

REFERENCES

1. Zhang L, Liu Y. Potential interventions for novel coronavirus in China: a systematic review. *J Med Virol.* (2020) 92:479–90. doi: 10.1002/jmv.25707
2. Tiberghien P, De Lambalerie X, Morel P, Gallian P, Lacombe K, Yazdanpanah Y. Collecting and evaluating convalescent plasma for COVID-19 treatment: why and how. *Vox Sang.* (2020). doi: 10.1111/vox.12926
3. Shen C, Wang Z, Zhao F, Yang Y, Li J, Yuan J, et al. Treatment of 5 Critically Ill Patients With COVID-19 With Convalescent Plasma. *JAMA*. (2020) 323:1582–9. doi: 10.1001/jama.2020.4783
4. Chen L, Xiong J, Bao L, Shi Y. Convalescent plasma as a potential therapy for COVID-19. *Lancet Infect Dis.* (2020) 20:398–400. doi: 10.1016/S1473-3099(20)30141-9
5. Casadevall A, Pirofski LA. The convalescent sera option for containing COVID-19. *J Clin Invest.* (2020) 130:1545–8. doi: 10.1172/JCI138003
6. Mair-Jenkins J, Saavedra-Campos M, Baillie JK, Cleary P, Khaw FM, Lim WS, et al. The effectiveness of convalescent plasma and hyperimmune immunoglobulin for the treatment of severe acute respiratory infections of viral etiology: a systematic review and exploratory meta-analysis. *J Infect Dis.* (2015) 211:80–90. doi: 10.1093/infdis/jiu396
7. Cheng Y, Wong R, Soo YO, Wong WS, Lee CK, Ng MH, et al. Use of convalescent plasma therapy in SARS patients in Hong Kong. *Eur J Clin Microbiol Infect Dis.* (2005) 24:44–6. doi: 10.1007/s10096-004-1271-9
8. Soo YO, Cheng Y, Wong R, Hui DS, Lee CK, Tsang KK, et al. Retrospective comparison of convalescent plasma with continuing high-dose methylprednisolone treatment in SARS patients. *Clin Microbiol Infect.* (2004) 10:676–8. doi: 10.1111/j.1469-0691.2004.00956.x
9. Yeh KM, Chiueh TS, Siu LK, Lin JC, Chan PK, Peng MY, et al. Experience of using convalescent plasma for severe acute respiratory syndrome among healthcare workers in a Taiwan hospital. *J Antimicrob Chemother.* (2005) 56:919–22. doi: 10.1093/jac/dki346
10. Ko JH, Seok H, Cho SY, Ha YE, Baek JY, Kim SH, et al. Challenges of convalescent plasma infusion therapy in Middle East respiratory coronavirus infection: a single centre experience. *Antivir Ther.* (2018) 23:617–22. doi: 10.3851/IMP3243
11. Duan K, Liu B, Li C, Zhang H, Yu T, Qu J, et al. Effectiveness of convalescent plasma therapy in severe COVID-19 patients. *Proc Natl Acad Sci USA.* (2020) 117:9490–6. doi: 10.1073/pnas.2004168117
12. Zhang B, Liu S, Tan T, Huang W, Dong Y, Chen L, et al. Treatment with convalescent plasma for critically ill patients with SARS-CoV-2 infection. *Chest.* (2020) 158:e9–13. doi: 10.1016/j.chest.2020.03.039
13. Ahn JY, Sohn Y, Lee SH, Cho Y, Hyun JH, Baek YJ, et al. Use of convalescent plasma therapy in two COVID-19 patients with acute respiratory distress syndrome in Korea. *J Korean Med Sci.* (2020) 35:e149. doi: 10.3346/jkms.2020.35.e149
14. Ye M, Fu D, Ren Y, Wang F, Wang D, Zhang F, et al. Treatment with convalescent plasma for COVID-19 patients in Wuhan, China. *J Med Virol.* (2020) 10.1002/jmv.25882. doi: 10.1002/jmv.25882
15. Us-Nih. *ClinicalTrials.gov| SARS-CoV 2 Convalescent Plasma*. National Institutes of Health (2020). Available: http://www.clinicaltrials.gov (accessed 18 June, 2020)
16. Lee JH, Kang ES, Kim DW. Two cases of transfusion-related acute lung injury triggered by HLA and anti-HLA antibody reaction. *J Korean Med Sci.* (2010) 25:1398–403. doi: 10.3346/jkms.2010.25.9.1398

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 07 August 2020
doi: 10.3389/fimmu.2020.01949

Overview of Immune Response During SARS-CoV-2 Infection: Lessons From the Past

Vibhuti Kumar Shah[1,2†], *Priyanka Firmal*[1,2†], *Aftab Alam*[2,3], *Dipyaman Ganguly*[3] *and Samit Chattopadhyay*[1,2,3*]

[1] Department of Biological Sciences, BITS Pilani, K. K. Birla Goa Campus, Goa, India, [2] National Centre for Cell Science, S. P. Pune University Campus, Pune, India, [3] Indian Institute of Chemical Biology, Kolkata, India

After the 1918 flu pandemic, the world is again facing a similar situation. However, the advancement in medical science has made it possible to identify that the novel infectious agent is from the coronavirus family. Rapid genome sequencing by various groups helped in identifying the structure and function of the virus, its immunogenicity in diverse populations, and potential preventive measures. Coronavirus attacks the respiratory system, causing pneumonia and lymphopenia in infected individuals. Viral components like spike and nucleocapsid proteins trigger an immune response in the host to eliminate the virus. These viral antigens can be either recognized by the B cells or presented by MHC complexes to the T cells, resulting in antibody production, increased cytokine secretion, and cytolytic activity in the acute phase of infection. Genetic polymorphism in MHC enables it to present some of the T cell epitopes very well over the other MHC alleles. The association of MHC alleles and its downregulated expression has been correlated with disease severity against influenza and coronaviruses. Studies have reported that infected individuals can, after recovery, induce strong protective responses by generating a memory T-cell pool against SARS-CoV and MERS-CoV. These memory T cells were not persistent in the long term and, upon reactivation, caused local damage due to cross-reactivity. So far, the reports suggest that SARS-CoV-2, which is highly contagious, shows related symptoms in three different stages and develops an exhaustive T-cell pool at higher loads of viral infection. As there are no specific treatments available for this novel coronavirus, numerous small molecular drugs that are being used for the treatment of diseases like SARS, MERS, HIV, ebola, malaria, and tuberculosis are being given to COVID-19 patients, and clinical trials for many such drugs have already begun. A classical immunotherapy of convalescent plasma transfusion from recovered patients has also been initiated for the neutralization of viremia in terminally ill COVID-19 patients. Due to the limitations of plasma transfusion, researchers are now focusing on developing neutralizing antibodies against virus particles along with immuno-modulation of cytokines like IL-6, Type I interferons (IFNs), and TNF-α that could help in combating the infection. This review highlights the similarities of the coronaviruses that caused SARS and MERS to the novel SARS-CoV-2 in relation to their pathogenicity and immunogenicity and also focuses on various treatment strategies that could be employed for curing COVID-19.

Keywords: coronavirus, immune response, COVID-19, T cells, MHC presentation, HLA, memory T cell

OPEN ACCESS

Edited by:
Alexis M. Kalergis, Pontificia Universidad Católica de Chile, Chile

Reviewed by:
Nilu Goonetilleke, University of North Carolina at Chapel Hill, United States
Perla Mariana Del Rio Estrada, Instituto Nacional de Enfermedades Respiratorias-México (INER), Mexico
Rachel Graham, University of North Carolina at Chapel Hill, United States

***Correspondence:**
Samit Chattopadhyay
samitc@goa.bits-pilani.ac.in; samitchatterji@yahoo.com

[†] These authors have contributed equally to this work

Specialty section:
This article was submitted to Viral Immunology, a section of the journal Frontiers in Immunology

Received: *18 April 2020*
Accepted: *20 July 2020*
Published: *07 August 2020*

Citation:
Shah VK, Firmal P, Alam A, Ganguly D and Chattopadhyay S (2020) Overview of Immune Response During SARS-CoV-2 Infection: Lessons From the Past. Front. Immunol. 11:1949. doi: 10.3389/fimmu.2020.01949

INTRODUCTION

The whole world is currently confronting a crisis situation that first appeared in late December 2019 as merely a few cases of pneumonia in Wuhan, China. The patients were exhibiting common symptoms like fever, dry cough, sore throat, breathlessness, and fatigue. Sample swabs from the oral cavity and anal region were collected along with the blood and Bronchoalveolar Lavage Fluid (BALF) from all seven of the patients, irrespective of their age and gender, which were then sent to the Wuhan Institute of Virology for further examination. As the outbreak initiated at the seafood market with the onset of winter, similar to that of the previous Severe Acute Respiratory Syndrome (SARS) infection, the scientists first screened the samples using pan-CoV qPCR primers. Surprisingly, five samples were reported positive for coronavirus. Thorough investigation employing next-generation sequencing and phylogenetic analysis led to the identification of the causative agent of this respiratory disease, a novel coronavirus (2019-nCoV) (1). As more cases started to appear around the world, on February 11, 2020, the World Health Organization assigned a name, **CO**rona **VI**rus **D**isease 20**19** or COVID-19, to the disease and declared it a pandemic on March 11, 2020. The virus was renamed from 2019-nCoV to SARS-CoV-2 by the International Committee on Taxonomy of Viruses on the basis of its genetic similarity to a previously known coronavirus, Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) (2). Transmission of SARS-CoV-2 occurs when a healthy individual inhales or comes into contact with respiratory droplets from an infected person. The average incubation period before patients exhibit disease symptoms ranges from 2 to 14 days (3). Before the spread of COVID-19, SARS emerged as an epidemic in 2003, followed by Middle East respiratory syndrome (MERS) in 2012, both caused by a novel coronavirus of zoonotic origin and assigned to the genus Betacoronavirus (4). The worldwide outbreak of SARS-CoV-2 has put life on hold, having a major impact on the world's economy, and has claimed ∼436,167 lives globally as of June 15, 2020 (5, 6). Unlike previous episodes of coronavirus spread, where it took months to identify the cause of infection and perform genome sequencing (7), advancement in science and technology made it possible to identify the causative organism swiftly. Within a few weeks of the outbreak, different laboratories across the world had sequenced the whole viral genome and had also provided structural and functional insights into the essential proteins required by the virus for its survival. These immediate scientific inputs helped with developing diagnostic kits and defining treatment strategies for effective prognosis and prevention (8–10). In this review, we are emphasizing the immunological aspect of SARS-CoV-2 pathogenesis by taking into consideration the previous experimental and clinical knowledge obtained from the coronaviruses that were responsible for causing SARS and MERS. This approach will assist in utilizing immunotherapies, repurposing the previously approved antiviral drugs, and developing therapeutic vaccines specific to novel coronavirus more effectively.

CLASSIFICATION AND COMPARISON OF SARS-CoV-2

Initial genome sequencing and phylogenetic analysis of novel coronavirus SARS-CoV-2 has shown that it is genetically similar to previously known coronavirus SARS-CoV and hence is placed under the family *Coronaviridae*. Coronavirus contains positive-sense single-stranded RNA (+ve ssRNA) as its genetic material, which can be about 30 kb in length and is mostly protected by an outer fatty layer of an envelope that also helps the virus to evade host immune response and assists its entry inside the host cell (11, 12). The subfamily *Coronavirinae* is further subdivided into four genera, namely alpha-, beta-, gamma-, and delta- coronavirus (α-CoV, β-CoV, γ-CoV, and δ-CoV). Viruses having the potential to infect humans are placed under the genus α-CoV and β-CoV (SARS-CoV & MERS-CoV), whereas viruses of γ-CoV and δ-CoV genera are mostly known to infect avians and pigs (13). The novel coronavirus, SARS-CoV-2 falls under the genus β-CoV, as it shares 88% sequence identity with SARS-CoV-like coronaviruses (derived from bat) but is only 79% identical to SARS-CoV and 50% identical to MERS-CoV (3). Thus, it can be deduced by its genome identity that the immediate host of this virus could be a bat, which then transmitted it to some unknown intermediate host that acted as a source for the transmission of the virus to humans.

Like those of SARS-CoV and MERS-CoV, the SARS-CoV-2 genome comprises of 12 open reading frames (ORFs) in number. At the 5′ end of the viral genome, overlapping ORFs 1a and 1b are present that encode the RNA polymerase and other non-structural proteins of the virus and occupy approximately two-thirds of the genome. Genes encoding structural proteins such as spike (S), membrane (M), envelope (E), and nucleocapsid (N), are present in the remaining one-third of its genome spanning from the 5′ to the 3′ terminal, along with several genes encoding non-structural proteins (NSPs) and accessory proteins scattered in between, as shown in **Figure 1**. Despite being in the same serogroup, there is a slight difference in the nucleotide number, sequence, gene order, and expression method among previously known coronaviruses and the novel SARS-CoV-2 (1, 14, 15). Recent reports highlight that a few amino acid substitutions have occurred in the novel coronavirus genes encoding the S protein, NSP2, NSP3, and receptor-binding domain (RBD). These mutations in the NSP2 & NSP3 are also believed to impart the enhanced infection abilities of the novel coronavirus (16, 17). RNA viruses are prone to acquiring genetic mutations that eventually help them to escape the host immune system and develop drug resistance. Researchers have also found minor mutations in SARS-CoV-2 genotype in different COVID-19 patients (18). One such hotspot of mutation in the SARS-CoV-2 genome is the RNA-dependent RNA polymerase gene. On analyzing 220 sequences across the globe, eight repetitive novel point mutations were observed. Viral genetic sequences accessed from Europe exhibited five mutation hotspots, whereas the remaining three point mutations were solely present in the sequences from North America. These unique mutations suggest that the viral strains are continuously evolving across the globe

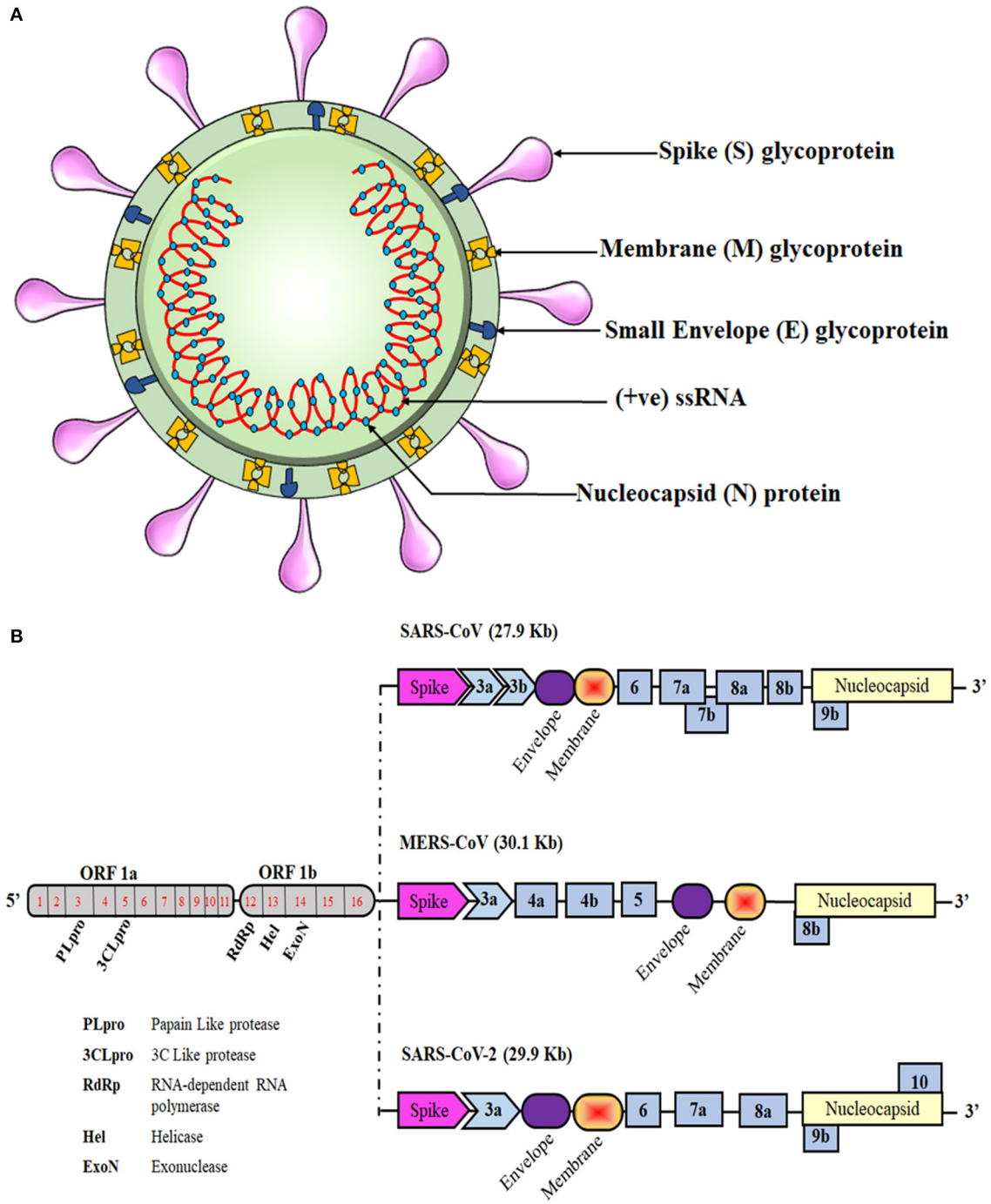


FIGURE 1 | Schematic representation of the coronavirus structure and genomic comparison of coronaviruses. **(A)** Representation of coronavirus showing different components of the particle, which is 100–160 nm in diameter. The single-stranded RNA (ssRNA) genome, covered with the envelope and membrane proteins, gains access into the host cell and hijacks the replication machinery. **(B)** The ssRNA of SARS-CoV-2 is about 30 kb and has similarities with the genomes of SARS-CoV and MERS-CoV. Translation of this ssRNA results in the formation of two polyproteins, namely pp1a and pp1ab, that are further sliced to generate numerous non-structural proteins (NSPs). The remaining ORFs encode for various structural and accessory proteins that help in assembly of the viral particle and evading immune response.

and that the strains from Europe, North America, and Asia might have co-existed the whole time (19). Another similar report analyzed 7,666 global viral genomic sequences and found 198 unique mutation sites on SARS-CoV-2 genome that encodes NSPs and S protein, suggesting that the virus is trying to adapt to its new host (20). As numerous drugs are currently being designed to target the proteins that are essential for the survival of the virus, rapid genetic mutation occurring in these proteins

might not prove to be a potential candidate for drug design. Therefore, the invariable region of the virus could be a better target to avoid drug failures.

Interestingly, SARS-CoV-2, similar to SARS-CoV, exploits the angiotensin-converting enzyme 2 (ACE2) receptor to gain access inside human cells, whereas MERS-CoV binds specifically to Dipeptidyl Peptidase 4 (DPP4) receptor (21, 22). Binding of the virus particle to the specific receptor on the host cell plays a key role in governing its pathogenicity. Functional evaluation was carried out to reveal the potential receptors for different Betacoronaviruses (β-CoV) including SARS-CoV-2, and it was found out that the entry of the virus particle was enhanced in human cells expressing ACE2 receptor instead of DPP4 or Aminopeptidase N (APN) in the case of the novel coronavirus (23). Recent structural insights provided by Cryo-EM studies of S protein in prefusion conformation highlighted that the binding efficiency of ACE2 and S protein of SARS-CoV-2 is 10–20 times greater than for the previously known SARS-CoV (24, 25). The latest reports suggest that the trimeric S protein of SARS-CoV-2 is sliced by transmembrane protease serine 2 (TMPRSS2), similar to SARS-CoV (26, 27). Hence, profound knowledge of the potential receptors to which the virus particle can bind and its associated proteases will help us in designing specific antiviral drugs and neutralizing antibodies and will lead us to foresee whether particular coronaviruses of zoonotic origin could be able to adapt and infect humans.

CORONAVIRUS REPLICATION

All coronaviruses initiate entry inside the target cell by engaging the host receptor with the S glycoprotein present on their surface so as to gain entry inside the target cell. The region of S protein containing the RBD is present on the S1 subunit. In a few coronaviruses, RBD is present at the N-terminus region of S1, whereas in SARS-CoV, it is situated at the C-terminus region (28, 29). The fusogenic activity of virus-cell membrane is governed by two tandem domains, heptad repeats (HR1,2) that are present on the S2 region of S protein (30, 31). Initially, it was believed that SARS-CoV enters the target cell merely by virtue of cell membrane integration of virus particle and host cell membrane (32). Later, it was discovered that an essential proteolytic cleavage event takes place in the S protein at the S2 position of SARS-CoV that results in membrane fusion and facilitates virus entry inside the cell (33).

Once the coronavirus is inside the host cell via membrane fusion, it releases its +ve ssRNA genome into the cytoplasmic compartment, where the translation of ORF-1a and ORF-1b begins resulting in the formation of two large polyproteins (pp1a and pp1ab). Three functional proteases then cleave the polyproteins into 16 non-structural proteins (NSP1-16), which eventually create the viral RNA polymerase and other accessory proteins for virus assembly (34–36). An uninterrupted replication-transcription event results in the formation of various nested sets of subgenomic (sg) mRNAs that eventually translate into numerous structural and accessory proteins (37). The E glycoproteins after synthesis are incorporated into the rough endoplasmic reticulum or Golgi membrane. The +ve ssRNA combines with capsid protein to form the nucleocapsid, followed by budding of assembled virus particles in the ER-Golgi Intermediate Compartment (ERGIC) (38). Lastly, the virus particle-loaded vesicles are fused with the cell membrane for effective shedding of the virus (4). These new virions are now accessible to infect the neighboring healthy cells and are also released into the surrounding environment via respiratory droplets that are highly contagious and hence potentially spread the disease to healthy individuals.

PATHOGENESIS OF COVID-19

The path followed by SARS-CoV-2 to reach the lungs is via the naso-oral cavity. Once the virus is inhaled, it enters the epithelial cells of the nasal cavity by engagement of ACE2 receptor with the viral RBD and initiates its replication (27, 39, 40). This initial asymptomatic phase lasts for about 1–2 days, during which the virus multiplies in the upper respiratory tract, where no major hindrance is caused by the innate immune cells. Within 2–14 days of initial encounter, the common symptoms of COVID-19 start to appear, which are similar to those of SARS and MERS, i.e., fever, dry cough, pharyngitis, shortness of breath, joint pain, and tiredness. Numerous problems arise during this phase of the disease, including nosocomial and fomite transmission of infection, which enhances the chances of community spread (41). Soon, the virus begins to move toward the lower respiratory tract via airways, and this triggers a strong innate immune response. Patients at this stage start exhibiting enhanced pro-inflammatory response that leads to viral sepsis accompanied by other complications, including pulmonary edema, Acute Respiratory Distress Syndrome (ARDS), different organ failures, and death in the worst scenarios (42). The infected individuals rarely show the intestinal symptoms like diarrhea that were evident in other coronavirus infections. Patients are recommended to be quarantined to prevent community spread of this pandemic virus (43). The severity of COVID-19 has been found to be greater in aged individuals and in people with a health history, such as those immune-compromised by HIV infection or by chemotherapy for cancer. Diabetic and asthma patients, along with individuals with hypertension, obesity, or heart, kidney, or liver disorders, are also at higher risk if they acquire the disease (44). Autopsy reports of individuals who died due to SARS show multi-organ dysfunction, with the highest viral titers in the lungs and immune cells in circulation, thus damaging the pulmonary and immune system (45, 46). As opposed to adults, only a very small population of children has been infected with SARS-CoV-2. In one study, the symptoms displayed by children above 15 years were found to be milder as compared to those of younger children, who showed severe symptoms but with rare deaths and better prognosis (47). The study speculated two major possibilities related to COVID-19 severity in children among different age groups. One of these rests on the finding that ACE2 activity is higher in children aged 4–13 years; after this age, it starts to decline until adolescence. This could be one of the reasons why lung fibrosis is observed mainly in younger children.

Secondly, differential $CD4^+$ and $CD8^+$ T cell populations have been seen in children as compared to adults (48, 49). A large number of clinical and epidemiological criteria were defined to assess probable pediatric cases of COVID-19 (50). A preliminary report from a cross-sectional study of children admitted to US and Canadian Pediatric Intensive Care Units (PICUs) during March 14-April 3, 2020, revealed that the 48 children were admitted in the USA whereas no COVID-19 cases were reported in Canadian PICUs. The study revealed that there are fewer COVID-19 cases in children as compared to adults and that there is a median PICU time of 5 days (51). A recent preprint from Paris reports that 11 children (age 3.7–16.6) were admitted experiencing symptoms similar to Kawasaki disease (KD) along with gastrointestinal issues and elevated inflammatory markers. Further investigation suggested that they were also SARS-CoV-2-positive, speculating that this could be the reason for KD shock syndrome (52). Similar cases have been observed in New York, where four otherwise healthy SARS-CoV-2-positive children started displaying symptoms similar to KD and toxic shock syndrome, thereby needing intensive care (53). Therefore, medical practitioners should be prepared to tackle such sudden post-infection complications to avoid the associated risks.

IMMUNE RESPONSE TO SARS-CoV-2

Once the virus gains access inside the target cell, the host immune system recognizes the whole virus or its surface epitopes, eliciting the innate or adaptive immune response (**Figure 2**). Pathogen recognition receptors (PRRs) present on immune cells, mainly Toll-like receptors 3, 7, and 8, are the first to identify the virus, which leads to enhanced interferon (IFN) production. The function of host innate immune cells is impaired during SARS-CoV and MERS-CoV infection by their non-structural proteins, which affects the overall cytokine production (54–56). Humoral response against SARS-CoV-2 has been found to be similar to that against other coronavirus infections, involving the characteristic IgG and IgM production. At the onset of SARS-CoV infection, B cells elicit an early response against the N protein, while antibodies against S protein could be detected after 4–8 days from the appearance of initial symptoms (57, 58). Although N protein is smaller than S protein, it is highly immunogenic, and the absence of glycosylation sites on it results in N-specific neutralizing antibody production at an early stage of acute infection (59). SARS-CoV-specific IgA, IgG, and IgM antibodies were detected after the onset of symptoms at different time points in infected patients. A persistent level of IgG was detected for a longer period, whereas IgM levels started to decline after 3 months (60, 61). In an observational case study of 16 SARS-CoV-2 patients, anti-S-RBD IgG was detected in all of the subjects, whereas anti-N IgG and anti-S-RBD IgM were detected in 15 patients and anti-N IgM in 14 patients (62). An ELISA-based time kinetics study to detect the COVID-19 specific humoral immune response showed that the patients produced IgM and IgG antibodies that did not cross-react with other human coronaviruses except SARS-CoV. IgM and IgA antibodies were detected 5 days after the onset of initial symptoms, whereas IgG was detected after 14 days (63). Another kinetic study of viral shedding and antibody detection was published in a preprint and reported the presence of higher IgG and IgM antibody titers in severe patients. They also observed that weak responders for IgG antibody had higher viral clearance than strong responders. This observation suggests that robust antibody response leads to disease severity while feeble response is associated with the elimination of virus (64). A case study on pediatric patients reports that 5 out of 6 children showed a protective humoral response, with neutralizing IgG and IgM antibodies targeting the N and S-RBD proteins of SARS-CoV-2 (65). These studies propose that IgM-based ELISA can be used for early diagnosis of patients along with qPCR techniques to improve the sensitivity and specificity of the technique.

In addition to neutralizing antibodies, which are defensive and useful, there are numerous non-neutralizing antibodies in the system that aid the infection of immune cells and APCs. Previously existing SARS-CoV antibodies may promote the viral infection in FcR-expressing cells (66). This ACE2-independent pathway of viral entry does not result in viral replication; rather, viral shedding by macrophages enhances inflammation and tissue injury by myeloid cell activation. This mechanism of viral entry through non-neutralizing antibody that results in aberrant activation of immune cells is called ADE (Antibody-Dependent Enhancement) (66, 67). ADE has been observed in a number of viral infections, including SARS and MERS. In the case of SARS, anti-S antibodies were observed to be involved in ADE to gain entry into FcR-expressing cells (68), while in MERS, a neutralizing Mab (Mersmab1) targeting RBD aided in MERS pseudo-virus entry via the DPP4 pathway (69). Although there is no clear evidence regarding ADE in SARS-CoV-2 infection, it is still necessary to consider all of the odds in the pursuit of developing vaccines and treatment regimens involving antibodies (70).

Antigen Presentation

During viral infection, T cells also recognize the viral antigens presented by MHC class I [MHC; Human Leukocyte Antigen (HLA) in humans], which in turn promotes the cytokine release and cytotoxic activity of $CD8^+$ T cells (71). But in some other cases, MHC class II is also found to present SARS-CoV peptides to $CD4^+$ T cells. Due to the genetic polymorphism of HLA, some haplotypes, like HLA-B*07, HLA-B*46, HLA-DRB1*12 (72), and HLA-Cw*08 (73), are found to be more susceptible to coronavirus infection, whereas the HLA-DRB1*03, HLA-A*02, and HLA-Cw*15 haplotypes are protected from SARS-CoV infection (74). Similarly, HLA-DRB1*11 and HLA-DQB1*02 were found to be vulnerable to MERS-CoV infection (75). Additionally, MHC expression is also found to be reduced during the infection due to epigenetic modifications of downstream molecules (76, 77). So far, HLA association is not very well-identified for SARS-CoV-2 infection, and this could be crucial for the prevention and treatment of COVID-19. However, in a recent report, blood plasma from COVID-19 patients was able to block the expression of HLA-DR on $CD14^+$ monocytes, which was restored effectively on inhibiting IL-6, suggesting that decreased HLA-DR expression in SARS-CoV-2 patients

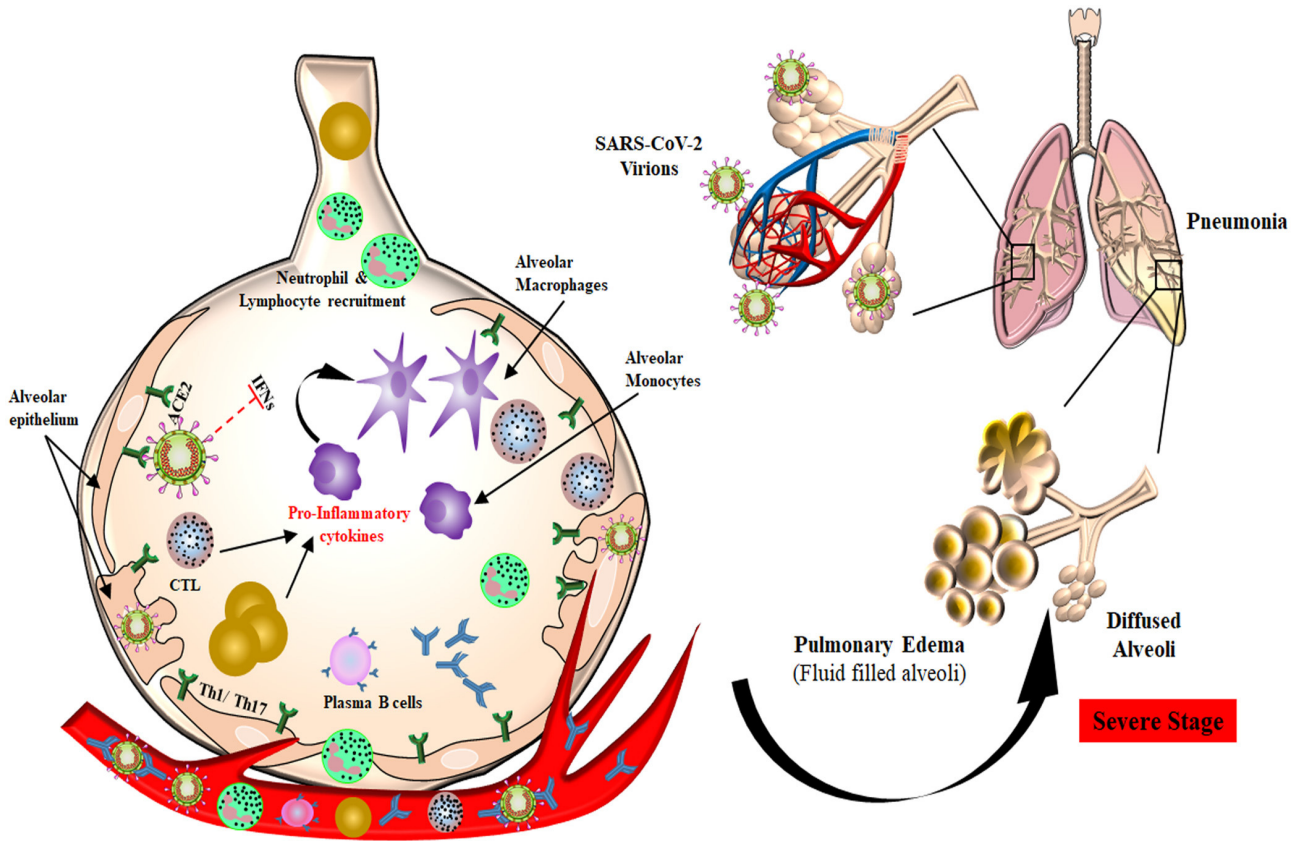


FIGURE 2 | Plausible host immune responses during COVID-19 infection. The SARS-CoV-2 virus infects through the naso-oral route, followed by infection in cells expressing ACE2 receptor in the lung, such as type 2 alveolar cells. These viruses dampen anti-viral IFN responses by evading the innate immune cells as a consequence of unrestrained virus replication. The infiltration of monocytes/macrophages, neutrophils, and several other adaptive immune cells leads to increased pro-inflammatory cytokines. In the helper T cell subset, stimulation of Th1/Th17 cells with viral epitopes may lead to aggravated inflammatory responses. This inflammatory response results in "cytokine storms" that lead to immunopathologies like pulmonary edema and pneumonia. Cytotoxic T cells recruited to the site of infection try to kill virus-infected cells in the lungs. B cells/plasma cells also recognize viral proteins and are activated to produce antibodies specific to SARS-CoV-2, which may help in deactivating viruses and provide systemic immunity in different organs.

is due to the buildup of hyper-inflammatory conditions (78). Decrease in MHC expression is also evident in cancer cells, which is a mechanism by which they evade the immune response by epigenetically modifying calnexin promoter. But infection with influenza virus in these cancer cells results in enhanced MHC-I presentation due to the increased expression of chromatin remodeling proteins, which stabilizes p53 expression and hence augments the immune surveillance of cancer cells (79). Therefore, molecules that can upregulate chromatin regulators and increase the MHC-I expression could potentially be used for COVID-19. Most of the T-cell epitopes presented by MHC complex are derived from structural proteins such as the S and N proteins of the coronavirus in both humans and animal models, while the NSPs have regulatory effects on the signaling cascade (80, 81). T cells can be stimulated by 14 epitopes, most of which are observed to be located on ORF3 and the S protein in SARS patients (61). In a large cohort study during SARS-CoV infection, S protein was the only immuno-dominant epitope for $CD8^+$ T-cell activation (61), whereas, in MERS, $CD8^+$ response was against the S and N proteins along with some of the M/E epitopes (82). These T-cell epitopes have been tested in animal models by assessing the lung pathology and T-cell response upon infection in BALB/c and C57BL/6 mice (80, 83). The sequence of SARS-CoV-2 being more similar to SARS-CoV than to MERS-CoV, with no mutation in 19 epitopes, provides a prospective subunit vaccine for stimulating a strong T-cell response in COVID 19 patients (84). In a recent study, samples from 20 convalescing COVID-19 patients were analyzed to check the development of adaptive immune response during infection. The results highlighted that helper T cells were eliciting a robust immune response against S, M, and N protein. The effect of adaptive immune response on humoral immunity was also compared, where a strong $CD4^+$ T-cell response against SARS-CoV-2 eventually resulted in an increase in anti-S-RBD-specific IgG and IgA antibody titer. Along with $CD4^+$ T cells, immunogenic epitopes on S, M, and N proteins were also able to activate $CD8^+$ T cells. However, such T-cell response was not specific to recovered patients only but was also present in 40–60%

of the individuals who were not exposed to SARS-CoV-2. Further analysis showed that they had pre-existing cross-reactive $CD4^+$ T cells, which might have been generated in response to some previous coronavirus infection. Hence, these T-cells could impart protective immunity in such individuals against SARS-CoV-2 to some extent (85). These epitopes could be a promising factor in developing immunotherapy by small molecules that can increase the presentation of viral epitopes.

Cytokine Production

A rapid and coordinated immune response during viral infection leads to enhanced secretion of various cytokines, which acts as a defense mechanism against the virus. Numerous reports suggest that individuals affected with SARS-CoV or MERS-CoV have dysregulated cytokine production from both innate and adaptive immune cells. In the case of SARS, infected hematopoietic cell, monocyte-macrophages, and other immune cells trigger enhanced secretion of pro-inflammatory cytokines like TNF-α, IL-6, and IFN-α/-γ, with reduced anti-inflammatory cytokines (86–88). Similarly, MERS-CoV infection leads to delayed but increased production of IFN-α and pro-inflammatory cytokines like IL-6, IL-8, and IL-1β (89–91). Such elevated levels of cytokines were associated with Multi-Organ Dysfunctional Syndrome (MODS) and ARDS due to the accumulation of numerous immune cells like macrophages, neutrophils, and dendritic cells in the lungs causing alveolar damage and edema (56, 92, 93). Similarly, in COVID-19 patients, secretion of cytokines and chemokines, which attract the immune cells to the lungs, was increased, hence causing ARDS, which is fatal to critically ill individuals (94, 95). Signature cytokines in severely ill COVID-19 patients were consistent with those in SARS and MERS, i.e., enhanced expression of IL-6, TNF-α, macrophage inflammatory protein 1-α (MIP-1α), MCP3, GM-CSF, IL-2, and IP-10 along with elevated chemokines (IP-10, CCL2/MCP1, CXCL1, CXCL5) were also detected in SARS-CoV-2 infection (96–99). In children, the increased inflammatory markers include IL-6, IL-1, and C-reactive protein along with procalcitonin in serum (52). In a case study, a 14-year-old child with cytokine storm was treated with anakinra (IL-1 receptor antagonist) in order to stabilize the respiratory illness and other clinical symptoms (100). Transcriptomic analysis of PBMC and BALF showed that a number of immune regulators were upregulated, particularly CXCL10, with respect to BALF. This study also reported that several apoptotic genes and P53 signaling molecules were upregulated, suggesting a possible reason for lymphopenia in these patients (101). Therapeutic measures to control such cytokines involve neutralizing antibodies or small molecular drugs that can stop the signaling cascade for cytokine production.

Immune Evasion

The most potent antiviral machinery acquired by immune cells is the secretion of interferons that act as secondary messengers stimulating the neighboring cells. Most innate immune cells are efficient in producing IFNs that are involved in obstructing cell proliferation, apoptosis, and immunomodulation (54, 102). As an escape mechanism, SARS-CoV or MERS-CoV uses several ways to overcome the host immune response, one of which is by severe leukopenia and lymphopenia (103–105). After gaining entry to the cell, these viruses encode different proteins that interact with downstream signaling molecules of TLRs and the JAK-STAT pathway. MERS-CoV encoded matrix protein, accessory proteins from ORF 4a, 4b, and 5, which directly inhibits the IFN promoter and nuclear localization of IRF3 (106). PLpro, encoded by SARS-CoV and MERS-CoV, prevents the dissociation of NF-κB from IκBα, whereas nonstructural proteins of SARS-CoV, i.e., PLpro and ORF3b, inhibit IRF3 phosphorylation and hence its translocation to the nucleus (4, 107, 108). These viral accessory proteins also inhibit the JAK-STAT pathway, resulting in inhibition of genes by ISRE promoters (109–111) (**Figure 3**). A new investigation revealed that SARS-CoV-2 infection leads to an overall decrease in the transcription of antiviral genes because of the lower production of Type I and III interferons with sufficient ISG expression, along with elevated chemokine secretion. Results obtained from *in-vivo and ex-vivo* COVID-19 experiments were in tune with the *in-vitro* findings. Therefore, a decrease in the innate antiviral response, along with hyper-inflammation, could be one of the causes of COVID-19 severity (112). In addition to reduction in T cells, SARS-CoV-2 infection also enhances the exhaustion of effector T cells, decreasing the immune response against the virus (94, 113). Exhaustion and loss in function of effector T cells is the result of increased expression of inhibitory receptors like PD-1, TIM-3, and TIGIT on its surface as a result of cytokines like IL-6, IL-10, and TNF-α or by decreasing the regulatory T-cell population (114, 115).

Memory T Cell

Following viral/antigen clearance, most of the effector T cell undergoes apoptosis in the contraction phase. Subsequently, a pool of memory T cells are generated that are programmed to fight against re-infection. $CD4^+$ memory T cells, upon re-stimulation, trigger B cells and other immune cells by cytokine production, while cytotoxic memory T cells help in destroying the infected cells during subsequent infection (116, 117). Case studies in recovered SARS patients showed that both $CD4^+$ and $CD8^+$ memory T cells were efficient in eliciting immune response from 3 months to 6 years without the presence of any antigens (118). In a case study of 23 recovered SARS-CoV patients, the patients showed very low frequencies of memory B cells, while memory T cells elicited a response against the S protein in 60% of recovered individuals (119). Considering the memory T-cell subset, N-specific helper T cells had more of central memory markers ($CD45RA^-$, $CCR7^+$, $CD62L^-$) while the $CD8^+$ T cell population had the effector memory ($CD45RA^+$, $CCR7^-$, $CD62L^-$) phenotype in a steady-state manner (120). The study suggests that an effective vaccine or T cell epitopes could be used to target a particular population for rapid viral clearance. In recent reports, COVID-19 subjects have shown reduced regulatory T cell populations and memory T cells, which may aggravate the inflammatory response leading to cytokine storm and hence enhance the tissue damage and organ failure (114). In a mouse model, the use of $CD4^+$ memory T cells as a vaccine by the intranasal, but not the subcutaneous, route imparted a protective response against the human coronavirus. The infused $CD4^+$ memory T cell, upon re-stimulation, produces IFN-γ and

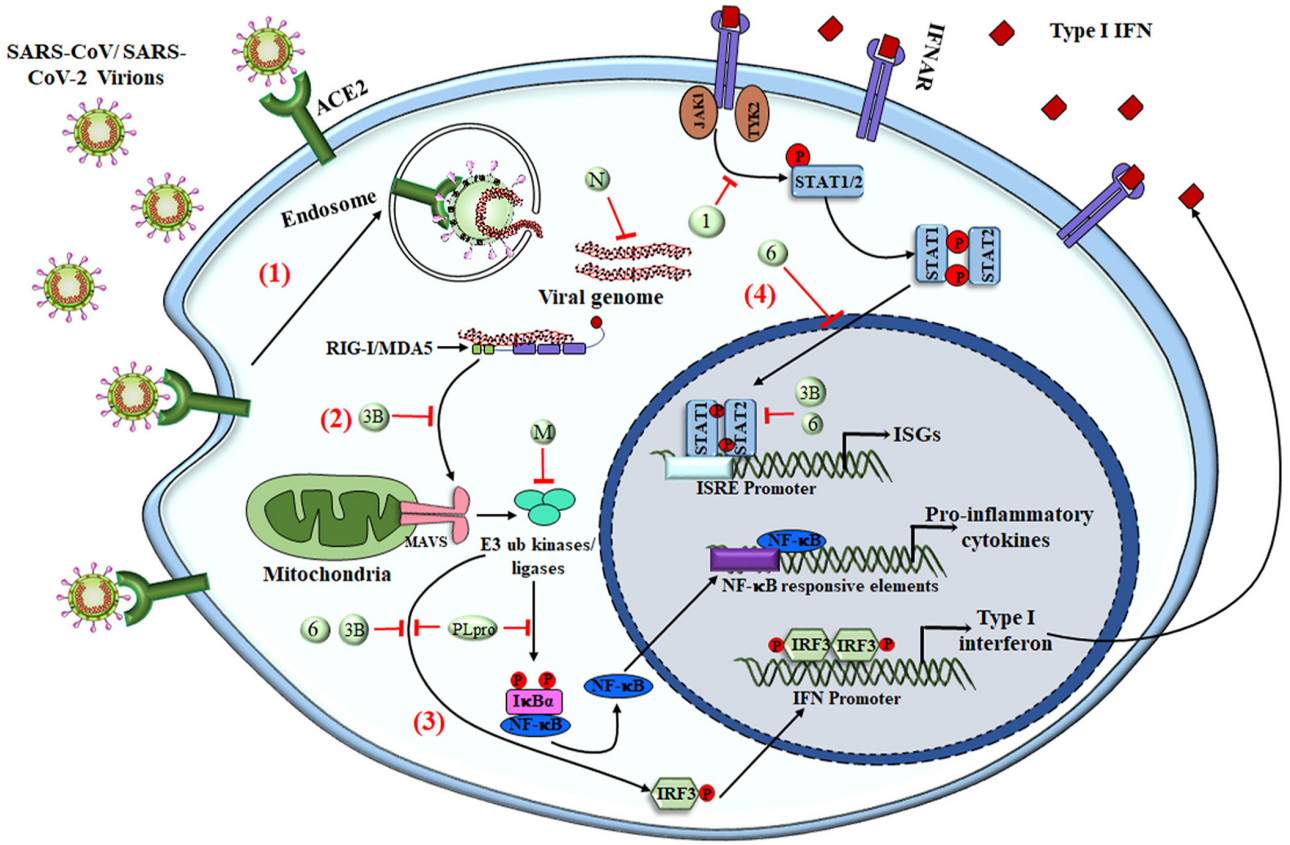


FIGURE 3 | SARS-CoV-mediated evasion of host innate immune response. The viral antigens are recognized via different PRRs to elicit the innate immune response. (1) Upon interaction of the virus with surface PRRs or the specific receptor, the particles are endocytosed into the cytosol and are then recognized by cytosolic PRRs like RIG-I and MDA5. (2) The viral genome, along with different proteins, interacts with MAVS and initiates NF-κB activation via triggering a signaling cascade that involves numerous E3 ubiquitin kinases and ligases. (3) Upon translocation into the nucleus, activated NF-κB acts as a transcriptional activator for numerous pro-inflammatory cytokines with an NF-κB-response element. The IFN-regulatory factor 3 (IRF3), upon phosphorylation via ubiquitin kinases, homodimerizes and moves inside the nucleus to activate the transcription of Type I IFNs. (4) Type I IFNs have both autocrine and paracrine mechanisms to activate the JAK–STAT signaling pathway via IFNα/β receptor (IFNAR), followed by phosphorylation of STAT1 and STAT2 via cytoplasmic protein JAK1 and TYK2 kinases. STAT1 & STAT2 heterodimers translocate into the nucleus and are recruited for transcription of the IFN-stimulated gene having an IFN-stimulated response element (ISRE) present on their promoter. SARS-CoV and other coronaviruses have found many ways to inhibit the signaling cascade by utilizing either the structural proteins (M and N protein) or NSPs (NSP1, NSP3b, and NSP6 along with PLpro), shown as numbers and letters in the figure. Together, the production of pro-inflammatory cytokines and type I IFNs tries to create an antiviral immune microenvironment that controls viral synthesis and infection, but the viruses have deployed various strategies to shut down these signaling pathways to counteract the immune response. RIG-I, Retinoic acid-Inducible Gene I protein; MDA5, Melanoma Differentiation-Associated protein 5; MAVS, Mitochondrial antiviral-signaling protein; M, Membrane protein; N, Nucleocapsid; IFNAR, IFNα/β receptor; ISGs, IFN-stimulated genes; ISRE, IFN-stimulated response element.

recruits $CD8^+$ T cells for rapid clearance in response to SARS-S366 peptide (121). Recently, a human ACE-2-expressing mouse model has been developed by CRISPR/Cas9 technology that recapitulates the human symptoms upon infection with SARS-CoV-2 through the intra-nasal route. This tool will be beneficial for evaluating the efficacy of vaccines for COVID-19 and also to study its transmission and pathogenesis (122).

TREATMENT STRATEGIES FOR COVID-19

Just like SARS and MERS, there are no specific clinically approved drugs available for COVID-19 as of June 15, 2020 (123). Currently, the treatment regime focuses mainly on providing intensive care in order to alleviate the symptoms and discomfort associated with COVID-19. Conservative fluid therapy accompanied by broad-spectrum antibiotics are also given to the patients as a protective measure to avoid opportunistic bacterial infections. However, ventilator support for respiration is provided to the patient under extreme conditions (124). Numerous FDA-approved antiviral drugs, vaccines, and immunotherapies that are already being used to treat other diseases have also been considered as a possible approach for treating COVID-19 (**Table 1**). But this approach may reduce the availability of these drugs and vaccines for the intended diseases and for the patients with the greatest need. The molecular, structural, and functional relationships of

TABLE 1 | List of drugs and vaccines for the treatment of COVID-19.

	Targets	Description	References
MONOCLONAL ANTIBODY THERAPY			
S230.15 mAbs m396 mAbs	RBD–ACE2 interaction	Tested in mice against SARS virus (strains Urbani, rGD03, or rSZ16).	(125)
MERS-4 MERS-27	RBD–DPP4 interaction	Blocks receptor–ligand interaction at the cell surface and prevents syncytia formation.	(126)
Tocilizumab	IL-6 receptor	Obstructs IL-6-mediated signal transduction.	(127)
Infliximab	TNF	Blocks soluble tumor necrosis factor and signal transduction, which helps maintain remission of COVID-19.	(128)
Adalimumab			
Lenzilumab	GM-CSF	Neutralization antibody for GM-CSF that is essential for chronic and acute inflammation in COVID-19.	(129)
Gimsilumab			(130)
Interferons	IFNß-1b	Enhances ISG expression via JAK/STAT signaling. Hinders virus multiplication and shedding.	(131)
	IFN- λ		(132)
SMALL-MOLECULE ANTIVIRAL DRUGS			
Aurine tricarboxylic acid	Viral RNA polymerase	Binds to viral polymerase, and tested against SARS virus in *in-vitro* culture.	(133)
Rupintrivir	Viral proteases	Protease inhibitor: inactivates 3CLpro and PLpro.	(134)
Benzopurpurin B	NSP15 endo-ribonuclease	Reduces viral infectivity of SARS virus in cell culture by inhibiting NSP15.	(135)
C-21	Angiotensin AT2 receptor	AT2 receptor agonist that may improve the viral damage to the lungs.	(134)
β-D-N4-hydroxycytidine (NHC)	Viral RNA polymerase	Inhibits replication of multiple coronaviruses. Can be used orally.	(136)
REPURPOSED FDA-APPROVED DRUGS			
Baricitinib	JAK kinase	Interferes with inflammatory signaling involving Janus kinase.	(137)
Lopinavir	Viral protease	Involved in immature, noninfectious HIV virus particle, and inhibits PLpro or 3CLpro in SARS-CoV-2.	(138)
Ritonavir	CYP3a (target unknown for coronavirus)	HIV protease inhibitor. No positive response in combination with lopinavir.	(139)
Favilavir	Viral RNA polymerase	Purine analog blocking viral RNA synthesis.	(140)
Remdesivir			(141)
Ribavirin		Guanosine nucleoside binds to nucleoside binding pocket of the enzyme.	(133, 140, 142)
Galidesivir		Adenosine analog, effective against Ebola, Zika, and other RNA viruses.	(143)
Chloroquine/hydroxychloroquine	Heme polymerase and ACE2	Increases endosomal pH and terminal glycosylation of ACE2, inhibiting SARS-CoV-2 entry.	(144, 145)
Nitazoxanide	Glutathione-S-transferase	Alters pH and inhibits viral maturation. Reported against TB, helminthic, and protozoan infection.	(140)
Umifenovir/arbidol	N/A	Interacts with aromatic residues of viral glycoproteins. Is being trialed for prophylactic action against COVID-19.	(146)

SARS-CoV-2 with SARS-CoV might define the use of existing anti-viral drugs against COVID-19 (147, 148), considering the total time it takes to perform clinical trials and get FDA approval for the use of novel drugs and vaccines. The increasing knowledge of the genetic, immunological, and molecular mechanisms behind its enhanced pathogenicity might help in developing specific treatment approaches for COVID-19 in the future.

Antiviral Agents

Considering the studies on the molecular mechanism of coronavirus infection (147), several antiviral drugs could be repurposed for the treatment of COVID-19. Remdesivir is a nucleotide analog that acts as an antiviral agent for a wide variety of viruses and has been tested widely against previous epidemics of coronavirus infections in both *in-vitro* and *in-vivo* models (138, 149–151). This adenosine analog

gets incorporated into the newly synthesized viral RNA, which inhibits the addition of further nucleotides by viral RNA-dependent RNA polymerase and hence terminates the ongoing transcription. Administration of intravenous remdesivir was found to be effective in treating the first known patient of COVID-19 in the USA (152). A randomized double-blinded clinical trial on 1,059 adult hospitalized COVID-19 patients was sponsored by the National Institute of Allergy and Infectious Diseases, USA, to further test the potency of intravenously administered remdesivir. The preliminary outcomes of the trial reported that remdesivir treatment decreased the median recovery time in the treatment group (11 days) as compared to the placebo group (15 days). The mortality rate was also less in the treatment group (7.1%) in contrast to the placebo group (11.9%) (153). Numerous clinical studies, similar to this, are required so as to validate the proposed drugs for COVID-19. Favipiravir, ribavirin, and galidesivir are also potential nucleoside analogs that might be useful against novel coronavirus infection (154). The combinatorial therapy approach of using remdesivir along with chloroquine, a well-known anti-malarial drug, has also been tested *in vitro* so as to study its effectiveness against SARS-CoV-2 (141, 155). It has been reported that chloroquine immuno-modulates the host microenvironment and also interferes with the replication of the virus and its interaction with the receptor (156, 157). In a randomized clinical trial (NCT04308668) involving 821 asymptomatic individuals across the US and Canada who had come into close contact with potential COVID-19 patients, the individuals were given either hydroxychloroquine or placebo as a prophylactic measure. The results revealed that hydroxychloroquine treatment had the same effect as did the placebo group. The usage of hydroxychloroquine resulted in minor side effects (40.1%) as compared to the placebo treatment (6.8%). However, no cardiovascular disorder or treatment-related major complications were observed (158). Based on the putative function of hydroxychloroquine on the endosomal acidification, whereby it is presumed to hinder viral uncapping, it can be observed that it has a great potential for prophylaxis, not to prevent infection but to reduce effective viral load in patients and thus lead to milder disease. Numerous clinical trials to further explore the usage of hydroxychloroquine in different combinations are in the pipeline and will finally provide a better understanding of the efficacy of this drug for COVID-19. A few anti-HIV drugs, such as lopinavir/ritonavir in combination with interferon beta (IFN-β), have been tested *in vivo* for treating coronavirus infections (SARS-CoV, MERS-CoV) and have also been used in the case of COVID-19 (138, 139, 159). Various complementary therapies could also be employed as a preventive measure against viral infections. Many essential proteases, such as chymotrypsin (3C-like protease) and PLpro, which are required by coronavirus for completing the replication process, can also be targeted using drugs. Cinanserin, flavonoids, and some small molecules are known to inhibit 3CLpro, whereas diarylheptanoids are used to inhibit PLpro (160–162). In a recent study, 16 potential anti-HCoV drugs were identified through a systems biology-based approach, such as melatonin, mercaptopurine, sirolimus, dactiomycin, and toremifene, which are to be tested further for their potency (163).

Plasma Therapy

In the absence of any dependable vaccine or drugs with tested efficacy and when the pandemic onslaught is ongoing, a worthy therapeutic approach is passive immunization using purified antibodies. The source of such antibodies could be the sera of convalescing individuals, mAbs, or genetically modified antibodies from an animal host, which can efficiently neutralize the virus. This is an age-old practice, with pioneering work having been done by the Nobel Laureate, Emil Behring, who applied this approach for diphtheria, and has been used whenever there are sudden outbreaks of viral diseases like SARS, MERS, H1N1, H5N1, Ebola, and many others (61, 164, 165). As opposed to active vaccination, plasma therapy is the only means to provide immediate immunity for viral clearance, as in the case of SARS-CoV-2. As in other epidemic diseases, convalescent sera are currently being employed for COVID-19 in a number of countries (166, 167). Although a randomized controlled trial is yet to be reported, limited studies in 10 patients have been documented with no remission of severe respiratory afflictions on receiving neutralizing antibodies from 39 convalesced donors with antibody titers of 1:160, along with drugs and oxygen support (168). A report from Hong Kong suggested that this therapy had poor outcome in SARS patients, with a number of limitations in their study (169). As with transfusion of any blood products, precautionary screening of infectious agent is warranted in plasma transfusion. Recently, the FDA in the USA has approved trials of convalescent plasma therapy in COVID-19 under specific guidelines; plasma donation is advised 3 weeks after a patient becomes virus-negative on PCR. The major challenge in this therapy is obtaining donors with similar blood antigens with a high antibody titer of SARS-CoV-2 (170). Another potential adverse effect of this approach is ADE of infection, which is common in so many other viruses. But, to date, the incidence of ADE has not been reported in the case of SARS-CoV-2. Another major point of contention is the selection of patients for this therapeutic approach. In most clinical trials, patients with severe diseases are being recruited, while the presumed mechanism of action of convalescent plasma, based on its content of virus-neutralizing antibodies, rather points to plausible favorable outcomes in earlier phases of the disease because in the later, more severe phases, the hyper-immune response, rather than the viral load, becomes the more critical pathology. Finally, there are no available data on the heterogeneity of response to convalescent plasma transfusion, which may further illustrate the importance of careful evidence-based patient selection, as heterogeneity of response may result from both virus and host-intrinsic factors which are, to date, not revealed.

Vaccine Design Strategies

Researchers around the world are working hard to develop a potential vaccine candidate so as to stop the deadly pandemic caused by SARS-CoV-2. However, vaccine development is not an easy task, as a number of successful clinical trials are required before approval for patients. Different approaches are being utilized for designing a specific vaccine targeting either the structural proteins or viral replication process, which

eventually results in the inhibition of viral growth and its further transmission. The common strategies involve the use of live attenuated vaccine (LAV), inactivated virus, subunit vaccines, monoclonal antibody vaccine, virus vectors, protein vaccines, and DNA/RNA-based vaccines (171–174). There are numerous subunit vaccines targeting all or a part of S protein that have already been tested for SARS and MERS in animal models (175) and could be potential candidates for testing against SARS-CoV-2. A recent pilot study with a purified inactivated SARS-CoV-2 virus vaccine displayed very promising outcomes in different animal models. The neutralizing antibodies generated after vaccination were able to effectively target 10 different strains of SARS-CoV-2 without developing any ADE of infection (176). Various randomized controlled trials (NCT04327206, NCT04328441) are also underway to evaluate the effectiveness of the BCG vaccine against SARS-CoV-2 for healthcare professionals. An adenovirus vector-based vaccine candidate, ChAdOx1 (presently AZD1222), developed by Oxford University (licensed to AstraZeneca) for use against SARS-CoV-2 has been reported to activate both the humoral and cell-mediated immune response when tested in rhesus monkey (177). The phase I clinical trial to confirm its potency is also in progress (NCT04324606). Another group has followed a similar approach by using a recombinant adenovirus type 5 (Ad5-nCoV) vector-based vaccine for COVID-19. The full report from the phase I clinical trial (NCT04313127) of Ad5-nCoV shows that it is very effective in generating both humoral and rapid T-cell response post immunization. The group is now ready for the next clinical trial phase to further strengthen the effectiveness of the Ad5-nCoV vaccine (178). It should be noted that there are potential risks associated with the usage of live attenuated viruses, for example, complications resulting in lung damage by infiltrating eosinophils, as seen in *in vivo* models (179, 180). However, eosinophil immunopathology due to SARS-CoV vaccine could be reduced by using TLR4 agonist as an adjuvant (181). Viral neutralizing antibodies specifically targeting various regions of S, i.e., S1-RBD, S1-NTD, or the S2 region, and blocking the interaction of virus with the receptor are well-known for SARS and MERS (182). These neutralizing antibodies could prove to be the best and potential candidate for cross-neutralization of SARS-CoV-2. Despite being structurally related, some of the SARS-CoV neutralizing monoclonal antibodies failed to interact with the S-protein of SARS-CoV-2, which could be attributable to the substantial differences in their RBD (183). A recent study reported the presence of high titres of neutralizing anti-S-RBD IgG antibodies, but no antibodies were detected against the N protein in recovered COVID-19 patients, suggesting that anti-S IgG persists longer than does anti-N IgG. Along with the humoral immune response, they also observed an S protein-specific T cell-population producing IFN-γ, which further contributes to conferring protective immunity against SARS-CoV-2 infection (184). Recently, a monoclonal antibody (47D11) has been identified from 51 SARS-Spike hybridomas that targets the conserved S-RBD region (residue 338–506) and therefore can very effectively neutralize SARS-CoV-2 along with SARS-CoV (185). On similar lines, a group has isolated a single-domain antibody from a phage display library targeting the S-RBD region of SARS-CoV-2. The fully humanized single-domain antibody was able to neutralize the virus by interacting with a cryptic epitope in S protein (186). These mAb and single-domain antibodies could be used to treat as well as to design quick diagnostic kits for COVID-19.

The new technology of the microneedle array (MNA) has been employed for delivering SARS-CoV-2 S1 subunit vaccine, which could be really helpful in the treatment of the emerging COVID-19 outbreak (187). The transfer of S1 subunit by MNA elicited a strong virus specific-antibody response in SARS-CoV-2 (187). A novel encapsulated mRNA vaccine candidate developed by ModernaTX, Inc. that encodes full length S protein of SARS-CoV-2, is also under clinical trial (NCT04283461). There is an urgent need to develop more such specific vaccines that could neutralize the novel coronavirus effectively (188).

Immunomodulatory Therapies

The host innate immune system encounters upcoming infections, and this results in elevated production of various cytokines and type I interferons (IFNs). In the case of prolonged infection, hyperactivation of the immune system may also result in the development of a pro-inflammatory microenvironment, leading to adverse outcomes and even death. The induction of numerous lymphokines, such as IL-6, IL-1β, TNF-α, and CCL2, that are pro-inflammatory in nature has also been observed in the case of COVID-19 (189–191). A previous study in a MERS animal model showed that treatment with recombinant type-1 IFN (rIFN) decreased the viral RNA level in lungs with a decrease in IFN-stimulating gene expression. Early treatment with rIFN resulted in a dampening of cytokine and chemokine release that lowered the migration of neutrophils and other cells in lung (91). An allogenic mesenchymal stem cell-based (Remestemcel-L) therapy developed by Mesoblast, which has been previously used for inflammatory conditions and graft vs. host disease in children and adults, is now being assessed for COVID-19 (192–194). In this therapy, bone marrow-derived MSCs from the donor are grown *in vitro* and are then transfused to the recipient patients. Upon infusion, these cells exhibit anti-inflammatory activity by reducing pro-inflammatory cytokine production via the recruitment of anti-inflammatory cells in the affected tissue (195). Currently, a randomized placebo-controlled trial (NCT04371393) with 300 patients is ongoing for treating ARDS caused by COVID-19. Treatment with rIFN, inhibitors of the pro-inflammatory pathway, cytokine inhibitors such as tocilizumab, lenzilumab, and many others are still to be used in combination with other drugs for treating COVID-19. So far, there is not much evidence from clinical trials of such inhibitors with which to predict the outcome of these anti-cytokine therapies.

CONCLUSION

Considering the current situation of more than 8 million people being infected, with ∼436,167 deaths as of June 15, 2020, there is an urgent need to control the SARS-CoV-2 pandemic. The fatality rate of SARS-CoV-2 in lower than those of other coronaviruses that caused catastrophes in the

past, but the higher infectivity rate makes it worse. Raising awareness of this contagious virus is one of the many ways by which its spread can be prevented. The governing authorities concerned in every country have approved guidelines and taken necessary action to quarantine infected people and break the chain of community spread. Antibodies, vaccines, and drugs developed for previously emerged coronaviruses could potentially be used for treating SARS-CoV-2. The combination of various neutralizing antibodies against S protein could enhance the effectiveness of viral clearance. Among various antivirals and other small molecules that are FDA approved, chloroquine/hydroxychloroquine has shown better positive outcome in COVID-19 patients. In clinical trials, some of the combinational antiviral drugs like lopinavir + ritonavir and blockers like angiotensin receptor blocker that were thought to be effective, have failed in curing the disease (139, 196). Cytokine storm being one of the symptoms of infected individuals, anti-cytokine therapy for TNF and IL-6 should be attempted to determine the efficacy of these antibodies in the treatment of SARS-CoV-2 infection. Clinical trial ChiCTR2000029765 with tocilizumab, a monoclonal humanized antibody against IL-6 receptor, has shown some efficacy, but this still needs to be tested in a larger cohort. With the increasing number of deaths, there is an immense need to accelerate the development of rapid and sensitive diagnostic kits and to commence clinical trials of the readily available and safe drugs to reduce the rising infections and COVID-19-related deaths so as to bring life back on track.

AUTHOR CONTRIBUTIONS

VS and PF contributed equally in writing the review. Conception of idea was done by SC, VS, and PF. Manuscript writing and editing was done by all the authors.

FUNDING

We are thankful to CSIR-Indian Institute of Chemical Biology, Jadavpur, Kolkata and National Centre for Cell Science (NCCS), Pune for providing infrastructure facilities. We would also like to acknowledge Department of Biotechnology-Systems Medicine Cluster (DBT-SyMeC) grant and J C Bose fellowship-SERB (Science and Engineering Research Board) to SC, for the financial support.

REFERENCES

1. Zhou P, Yang X-L, Wang XG, Hu B, Zhang L, Zhang W, et al. A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature.* (2020) 579:270–3. doi: 10.1038/s41586-020-2012-7
2. Lu R, Zhao X, Li J, Niu P, Yang B, Wu H, et al. Genomic characterisation and epidemiology of 2019 novel coronavirus: implications for virus origins and receptor binding. *Lancet.* (2020) 395:565–74. doi: 10.1016/S0140-6736(20)30251-8
3. Ong SWX, Tan YK, Chia PY, Lee TH, Ng OT, Wong MSY, et al. Air, surface environmental, and personal protective equipment contamination by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) from a symptomatic patient. *JAMA - J Am Med Assoc.* (2020) 323:5–7. doi: 10.1001/jama.2020.3227
4. de Wit E, van Doremalen N, Falzarano D, Munster VJ. SARS and MERS : recent insights into emerging coronaviruses. *Nat Rev Microbiol.* (2016) 14:523–34. doi: 10.1038/nrmicro.2016.81
5. Verity R, Okell LC, Dorigatti I, Winskill P, Whittaker C, Imai N, et al. Estimates of the severity of coronavirus disease 2019 : a model-based analysis. *Lancet Infect Dis.* (2020) 3099:1–9. doi: 10.1016/S1473-3099(20)30243-7
6. *COVID-19 : Disrupting Lives, Economies and Societies*. Vol. 136. United Nation, Department of Economic and Social Affairs (2020). p. 1–4. Available online at: https://www.un.org/development/desa/dpad/publication/world-economic-situation-and-prospects-april-2020-briefing-no-136/
7. Marra MA, Jones SJM, Astell CR, Holt RA, Brooks-wilson A, Butterfield YSN, et al. The genome sequence of the sars-associated coronavirus. *Science.* (2003) 300:1399–404. doi: 10.1126/science.1085952
8. Wu F, Zhao S, Yu B, Chen YM, Wang W, Song ZG, et al. A new coronavirus associated with human respiratory disease in China. *Nature.* (2020) 579:265–9. doi: 10.1038/s41586-020-2008-3
9. Zhang L, Lin D, Sun X, Curth U, Drosten C, Sauerhering L, et al. Crystal structure of SARS-CoV-2 main protease provides a basis for design of improved α-ketoamide inhibitors. *Science.* (2020) 3405:1–9. doi: 10.1126/science.abb3405
10. Yan R, Zhang Y, Li Y, Xia L, Guo Y, Zhou Q. Structural basis for the recognition of the SARS-CoV-2 by full-length human ACE2. *Science.* (2020) 2:1444–8. doi: 10.1126/science.abb2762
11. Zhu N, Zhang D, Wang W, Li X, Yang B, Song J, et al. A novel coronavirus from patients with pneumonia in China, 2019. *N Engl J Med.* (2020) 382:727–33. doi: 10.1056/NEJMoa2001017
12. Schoeman D, Fielding BC. Coronavirus envelope protein: current knowledge. *Virol J.* (2019) 16:1–22. doi: 10.1186/s12985-019-1182-0
13. Lim Y, Ng Y, Tam J, Liu D. Human coronaviruses: a review of virus–host interactions. *Diseases.* (2016) 4:26. doi: 10.3390/diseases4030026
14. Cui J, Li F, Shi ZL. Origin and evolution of pathogenic coronaviruses. *Nat Rev Microbiol.* (2019) 17:181–92. doi: 10.1038/s41579-018-0118-9
15. Song Z, Xu Y, Bao L, Zhang L, Yu P, Qu Y, et al. From SARS to MERS, thrusting coronaviruses into the spotlight. *Viruses.* (2019) 11:59. doi: 10.3390/v11010059
16. Wu A, Peng Y, Huang B, Ding X, Wang X, Niu P, et al. Genome composition and divergence of the novel coronavirus (2019-nCoV) originating in China. *Cell Host Microbe.* (2020) 27:325–8. doi: 10.1016/j.chom.2020.02.001
17. Angeletti S, Benvenuto D, Bianchi M, Giovanetti M, Pascarella S, Ciccozzi M. COVID-2019: The role of the nsp2 and nsp3 in its pathogenesis. *J Med Virol.* (2020) 92:1–5. doi: 10.1002/jmv.25719
18. Zhang L, Shen F, Chen F, Lin Z. Origin and evolution of the 2019 novel coronavirus. *Clin Infect Dis.* (2020) 71:882–3. doi: 10.1093/cid/ciaa112
19. Pachetti M, Marini B, Benedetti F, Giudici F, Mauro E, Storici P, et al. Emerging SARS-CoV-2 mutation hot spots include a novel RNA-dependent-RNA polymerase variant. *J Transl Med.* (2020) 18:1–9. doi: 10.1186/s12967-020-02344-6
20. van Dorp L, Acman M, Richard D, Shaw LP, Ford CE, Ormond L, et al. Emergence of genomic diversity and recurrent mutations in SARS-CoV-2. *Infect Genet Evol.* (2020) 83:104351. doi: 10.1016/j.meegid.2020.104351
21. Li W, Moore MJ, Vasilieva N, Sui J, Wong SK, Berne MA, et al. Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus Wenhui. *Nature.* (2003) 426:450–4. doi: 10.1038/nature02145
22. Raj VS, Mou H, Smits SL, Dekkers DHW, Müller MA, Dijkman R, et al. Dipeptidyl peptidase 4 is a functional receptor for the emerging human coronavirus-EMC. *Nature.* (2013) 495:251–4. doi: 10.1038/nature12005
23. Letko M, Marzi A, Munster V. Functional assessment of cell entry and receptor usage for SARS-CoV-2 and other lineage B betacoronaviruses. *Nat Microbiol.* (2020) 5:562–9. doi: 10.1038/s41564-020-0688-y

24. Song W, Gui M, Wang X, Xiang Y. Cryo-EM structure of the SARS coronavirus spike glycoprotein in complex with its host cell receptor ACE2. *PLoS Pathog.* (2018) 14:1–19. doi: 10.1371/journal.ppat.1007236
25. Wrapp D, Wang N, Corbett KS, Goldsmith JA, Hsieh CL, Abiona O, et al. Cryo-EM structure of the 2019-nCoV spike in the prefusion conformation. *Science.* (2020) 367:1260–3. doi: 10.1126/science.aax0902
26. Millet JK, Whittaker GR. Host cell proteases: critical determinants of coronavirus tropism and pathogenesis. *Virus Res.* (2015) 202:120–34. doi: 10.1016/j.virusres.2014.11.021
27. Hoffmann M, Kleine-Weber H, Schroeder S, Krüger N, Herrler T, Erichsen S, et al. SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor. *Cell.* (2020) 181:1–10. doi: 10.1016/j.cell.2020.02.052
28. Taguchi F, Kubo H, Suzuki H, Yamada YK. Localization of neutralizing epitopes and receptor-binding site in murine coronavirus spike protein. *Adv Exp Med Biol.* (1995) 380:359–65. doi: 10.1007/978-1-4615-1899-0_58
29. Cheng PKC, Wong DA, Tong LKL, Ip SM, Lo ACT, Lau CS, et al. Viral shedding patterns of coronavirus in patients with probable severe acute respiratory syndrome. *Lancet.* (2004) 363:1699–700. doi: 10.1016/S0140-6736(04)16255-7
30. Zhang N, Jiang S, Du L. Current advancements and potential strategies in the development of MERS-CoV vaccines. *Expert Rev Vaccines.* (2014) 13:761–74. doi: 10.1586/14760584.2014.912134
31. Xia S, Zhu Y, Liu M, Lan Q, Xu W, Wu Y, et al. Fusion mechanism of 2019-nCoV and fusion inhibitors targeting HR1 domain in spike protein. *Cell Mol Immunol.* (2020) 17:3–5. doi: 10.1038/s41423-020-0374-2
32. Simmons G, Reeves JD, Rennekamp AJ, Amberg SM, Piefer AJ, Bates P. Characterization of severe acute respiratory syndrome-associated coronavirus (SARS-CoV) spike glycoprotein-mediated viral entry. *Proc Natl Acad Sci USA.* (2004) 101:4240–5. doi: 10.1073/pnas.0306446101
33. Belouzard S, Chu VC, Whittaker GR. Activation of the SARS coronavirus spike protein via sequential proteolytic cleavage at two distinct sites. *Proc Natl Acad Sci USA.* (2009) 106:5871–6. doi: 10.1073/pnas.0809524106
34. Brockway SM, Clay CT, Lu XT, Denison MR. Characterization of the expression, intracellular localization, and replication complex association of the putative mouse hepatitis virus RNA-dependent RNA polymerase. *J Virol.* (2003) 77:10515–27. doi: 10.1128/jvi.77.19.10515-10527.2003
35. Sawicki SG, Sawicki DL. Coronavirus transcription: a perspective. *Curr Top Microbiol Immunol.* (2005) 287:31–55. doi: 10.1007/3-540-26765-4_2
36. Adriaan H. de Wilde, Eric J, Snijder MK, van HMJ. Host factors in coronavirus replication. *Curr Top Microbiol Immunol.* (2018) 419:1–42. doi: 10.1007/82_2017_25
37. Hussain S, Pan J, Chen Y, Yang Y, Xu J, Peng Y, et al. Identification of novel subgenomic RNAs and noncanonical transcription initiation signals of severe acute respiratory syndrome coronavirus. *J Virol.* (2005) 79:5288–95. doi: 10.1128/jvi.79.9.5288-5295.2005
38. Perrier A, Bonnin A, Desmarets L, Danneels A, Goffard A, Rouillé Y, et al. The C-terminal domain of the MERS coronavirus M protein contains a trans-Golgi network localization signal. *J Biol Chem.* (2019) 294:14406–21. doi: 10.1074/jbc.RA119.008964
39. Walls AC, Park YJ, Tortorici MA, Wall A, McGuire AT, Veesler D. Structure, function, and antigenicity of the SARS-CoV-2 spike glycoprotein. *Cell.* (2020) 180:281–92. doi: 10.1016/j.cell.2020.02.058
40. Sungnak W, Huang N, Bécavin C, Berg M, Queen R, Litvinukova M, et al. SARS-CoV-2 entry factors are highly expressed in nasal epithelial cells together with innate immune genes. *Nat Med.* (2020) 26:681–7. doi: 10.1038/s41591-020-0868-6
41. CDC. Information for Healthcare Professionals: COVID-19 Underlying Conditions. (2020). Available online at: https://www.cdc.gov/coronavirus/2019-ncov/hcp/underlying-conditions.html (accessed April 6, 2020).
42. CDC. Infection Control: severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). (2020). Available online at: https://www.cdc.gov/coronavirus/2019-ncov/hcp/infection-control-recommendations.html (accessed April 8, 2020).
43. Prompetchara E, Ketloy C, Palaga T. Immune responses in COVID-19 and potential vaccines: lessons learned from SARS and MERS epidemic. *Asian Pacific J allergy Immunol.* (2020) 38:1–9. doi: 10.12932/AP-200220-0772
44. CDC. People Who Are at Higher Risk for Severe Illness | Coronavirus | COVID-19. (2020). Available online at: https://www.cdc.gov/coronavirus/2019-ncov/need-extra-precautions/groups-at-higher-risk.html (accessed April 7, 2020).
45. Mazzulli T, Farcas GA, Poutanen SM, Willey BM, Low DE, Butany J, et al. Severe acute respiratory syndrome-associated coronavirus in lung tissue. *Emerg Infect Dis.* (2004) 10:20–4. doi: 10.3201/eid1001.030404
46. Gu J, Gong E, Zhang B, Zheng J, Gao Z, Zhong Y, et al. Multiple organ infection and the pathogenesis of SARS. *J Exp Med.* (2005) 202:415–24. doi: 10.1084/jem.20050828
47. Centis R, Sc M. SARS-CoV-2 Infection in Children. *N Engl J Med.* (2020) 382:4–6. doi: 10.1056/NEJMc1210001
48. Zhang Z, Guo L, Lu X, Zhang C, Wang X, Huang L, et al. Vulnerability of children with COVID-19 infection and ACE2 profiles in lungs. *SSRN.* (2020) 1:1–18. doi: 10.2139/ssrn.3602441
49. Zhu L, Lu X, Chen L. Possible causes for decreased susceptibility of children to coronavirus. *Pediatr Res.* (2020) 145:2020. doi: 10.1038/s41390-020-0892-8
50. Chen ZM, Fu JF, Shu Q, Chen YH, Hua CZ, Li FB, et al. Diagnosis and treatment recommendations for pediatric respiratory infection caused by the 2019 novel coronavirus. *World J Pediatr.* (2020) 16:240–6. doi: 10.1007/s12519-020-00345-5
51. Shekerdemian LS, Mahmood NR, Wolfe KK, Riggs BJ, Ross CE, McKiernan CA, et al. Characteristics and outcomes of children with coronavirus disease 2019 (COVID-19) infection admitted to US and canadian pediatric intensive care units. *JAMA Pediatr.* (2020) 2019:1–6. doi: 10.1001/jamapediatrics.2020.1948
52. Toubiana J, Poirault C, Corsia A, Bajolle F, Fourgeaud J, Angoulvant F, et al. Outbreak of Kawasaki disease in children during COVID-19 pandemic: a prospective observational study in Paris, France. *medRxiv.* (2020) 1–21. doi: 10.1101/2020.05.10.20097394
53. Waltuch T, Gill P, Zinns LE, Whitney R, Tokarski J, Tsung JW, et al. Features of COVID-19 post-infectious cytokine release syndrome in children presenting to the emergency department. *Am J Emerg Med.* (2020). doi: 10.1016/j.ajem.2020.05.058. [Epub ahead of print].
54. Le Bon A, Tough DF. Links between innate and adaptive immunity via type I interferon. *Curr Opin Immunol.* (2002) 14:432–6. doi: 10.1016/S0952-7915(02)00354-0
55. Thiel V, Weber F. Interferon and cytokine responses to SARS-coronavirus infection. *Cytokine Growth Factor Rev.* (2008) 19:121–32. doi: 10.1016/j.cytogfr.2008.01.001
56. Hu W, Yen YT, Singh S, Kao CL, Wu-Hsieh BA. SARS-CoV regulates immune function-related gene expression in human monocytic cells. *Viral Immunol.* (2012) 25:277–88. doi: 10.1089/vim.2011.0099
57. Tan YJ, Goh PY, Fielding BC, Shen S, Chou CF, Fu JL, et al. Profiles of antibody responses against severe acute respiratory syndrome coronavirus recombinant proteins and their potential use as diagnostic markers. *Clin Diagn Lab Immunol.* (2004) 11:362–71. doi: 10.1128/CDLI.11.2.362-371.2004
58. Wu HS, Hsieh YC, Su IJ, Lin TH, Chiu SC, Hsu YF, et al. Early detection of antibodies against various structural proteins of the SARS-associated coronavirus in SARS patients. *J Biomed Sci.* (2004) 11:117–26. doi: 10.1159/000075294
59. Meyer B, Drosten C, Müller MA. Serological assays for emerging coronaviruses : challenges and pitfalls. *Virus Res.* (2014) 194:175–83. doi: 10.1016/j.virusres.2014.03.018
60. Li Gang, Chen Xuejuan AX. Profile of specific antibodies to the SARS-associated coronavirus. *N Engl J Med.* (2003) 349:508–9. doi: 10.1056/NEJM200307313490520
61. Ka-fai Li C, Wu H, Yan H, Ma S, Wang L, Zhang M, et al. T cell responses to whole SARS coronavirus in humans. *J Immunol.* (2008) 181:5490–500. doi: 10.4049/jimmunol.181.8.5490
62. To KK, Tak O, Tsang Y, Leung W, Tam AR, Wu T, et al. Temporal profiles of viral load in posterior oropharyngeal saliva samples and serum antibody

responses during infection by SARS-CoV-2 : an observational cohort study. *Lancet Infect Dis.* (2020) 20:565–74. doi: 10.1016/S1473-3099(20)30196-1
63. Guo L, Ren L, Yang S, Xiao M, Chang D, Yang F, et al. Profiling early humoral response to diagnose novel coronavirus disease (COVID-19). *Clin Infect Dis.* (2020) 21:1–8. doi: 10.1093/cid/ciaa310
64. Tan W, Lu Y, Zhang J, Wang J, Dan Y, Tan Z, et al. Viral kinetics and antibody responses in patients with COVID-19. *medRxiv.* (2020) 2020.03.24.20042382. doi: 10.1101/2020.03.24.20042382
65. Zhang Y, Xu J, Jia R, Yi C, Gu W, Liu P, et al. Protective humoral immunity in SARS-CoV-2 infected pediatric patients. *Cell Mol Immunol.* (2020) 17:2–4. doi: 10.1038/s41423-020-0438-3
66. Iwasaki A, Yang Y. The potential danger of suboptimal antibody responses in COVID-19. *Nat Rev Immunol.* (2020) 20:1–3. doi: 10.1038/s41577-020-0321-6
67. Ricke DO, Malone RW. Medical countermeasures analysis of 2019-nCoV and vaccine risks for antibody-dependent enhancement (ADE). *Preprint.* (2020) 1–24. doi: 10.20944/preprints202003.0138.v1
68. Yip M, Leung H, Li P, Cheung C, Dutry I, Li D, et al. Antibody-dependent enhancement of SARS coronavirus infection and its role in the pathogenesis of SARS. *Hong Kong Med J.* (2016) 22:25–31.
69. Wan Y, Shang J, Sun S, Tai W, Chen J, Geng Q, et al. Molecular mechanism for antibody-dependent enhancement of coronavirus entry. *J Virol.* (2020) 94:1–15. doi: 10.1128/JVI.02015-19
70. Peeples L. Avoiding pitfalls in the pursuit of a COVID-19 vaccine. *Proc Natl Acad Sci USA.* (2020) 117:4–7. doi: 10.1073/pnas.2005456117
71. Liu J, Wu P, Gao F, Qi J, Kawana-Tachikawa A, Xie J, et al. Novel immunodominant peptide presentation strategy: a featured HLA-A*2402-restricted cytotoxic t-lymphocyte epitope stabilized by intrachain hydrogen bonds from severe acute respiratory syndrome coronavirus nucleocapsid protein. *J Virol.* (2010) 84:11849–57. doi: 10.1128/jvi.01464-10
72. Keicho N, Itoyama S, Kashiwase K, Phi NC, Long HT, Ha LD, et al. Association of human leukocyte antigen class II alleles with severe acute respiratory syndrome in the Vietnamese population. *Hum Immunol.* (2009) 70:527–31. doi: 10.1016/j.humimm.2009.05.006
73. Chen YMA, Liang SY, Shih YP, Chen CY, Lee YM, Chang L, et al. Epidemiological and genetic correlates of severe acute respiratory syndrome coronavirus infection in the hospital with the highest nosocomial infection rate in Taiwan in 2003. *J Clin Microbiol.* (2006) 44:359–65. doi: 10.1128/JCM.44.2.359-365.2006
74. Wang SF, Chen KH, Chen M, Li WY, Chen YJ, Tsao CH, et al. Human-leukocyte antigen class i Cw 1502 and Class II DR 0301 genotypes are associated with resistance to severe acute respiratory syndrome (SARS) infection. *Viral Immunol.* (2011) 24:421–6. doi: 10.1089/vim.2011.0024
75. Hajeer AH, Balkhy H, Johani S, Yousef MZ, Arabi Y. Association of human leukocyte antigen class II alleles with severe Middle East respiratory syndrome-coronavirus infection. *Ann Thorac Med.* (2016) 11:211–3. doi: 10.4103/1817-1737.185756
76. Josset L, Menachery VD, Gralinski LE, Agnihothram S, Sova P, Carter VS, et al. Cell host response to infection with novel human coronavirus EMC predicts potential antivirals and important differences with SARS coronavirus. *MBio.* (2013) 4:e00165–13. doi: 10.1128/mBio.00165-13
77. Menachery VD, Schäfer A, Burnum-Johnson KE, Mitchell HD, Eisfeld AJ, Walters KB, et al. MERS-CoV and H5N1 influenza virus antagonize antigen presentation by altering the epigenetic landscape. *Proc Natl Acad Sci USA.* (2018) 115:E1012–21. doi: 10.1073/pnas.1706928115
78. Giamarellos-bourboulis EJ, Netea MG, Rovina N, Koulouris N, Gogos C, Koutsoukou A, et al. Complex immune dysregulation in COVID-19 patients with severe respiratory failure. *Cell Host Microbe.* (2020) 27:1–9. doi: 10.1016/j.chom.2020.04.009
79. Alam A, Taye N, Patel S, Thube M, Mullick J, Shah VK, et al. SMAR1 favors immunosurveillance of cancer cells by modulating calnexin and MHC I expression. *Neoplasia (United States).* (2019) 21:945–62. doi: 10.1016/j.neo.2019.07.002
80. Zhao J, Zhao J, Perlman S. T cell responses are required for protection from clinical disease and for virus clearance in severe acute respiratory syndrome coronavirus-infected mice. *J Virol.* (2010) 84:9318–25. doi: 10.1128/jvi.01049-10
81. Huang J, Cao Y, Du J, Bu X, Ma R, Wu C. Priming with SARS CoV S DNA and boosting with SARS CoV S epitopes specific for CD4+ and CD8+ T cells promote cellular immune responses. *Vaccine.* (2007) 25:6981–91. doi: 10.1016/j.vaccine.2007.06.047
82. Zhao J, Alshukairi AN, Baharoon SA, Ahmed WA, Bokhari AA, Nehdi AM, et al. Recovery from the Middle East respiratory syndrome is associated with antibody and T cell responses. *Sci Immunol.* (2017) 2:1–11. doi: 10.1126/sciimmunol.aan5393
83. Gupta V, Tabiin TM, Sun K, Chandrasekaran A, Anwar A, Yang K, et al. SARS coronavirus nucleocapsid immunodominant T-cell epitope cluster is common to both exogenous recombinant and endogenous DNA-encoded immunogens. *Virology.* (2006) 347:127–39. doi: 10.1016/j.virol.2005.11.042
84. Ahmed SF, Quadeer AA, McKay MR. Preliminary identification of potential vaccine targets for the COVID-19 Coronavirus (SARS-CoV-2) Based on SARS-CoV immunological studies. *Viruses.* (2020) 12, 1–15. doi: 10.1101/2020.02.03.933226
85. Grifoni A, Weiskopf D, Ramirez SI, Mateus J, Jennifer M, Moderbacher CR, et al. Targets of T cell responses to SARS-CoV-2 coronavirus in humans with COVID-19 disease and unexposed individuals. *Cell.* (2020) 181:1489–501. doi: 10.1016/j.cell.2020.05.015
86. Cameron MJ, Bermejo-Martin JF, Danesh A, Muller MP, Kelvin DJ. Human immunopathogenesis of severe acute respiratory syndrome (SARS). *Virus Res.* (2008) 133:13–9. doi: 10.1016/j.virusres.2007.02.014
87. Wong CK, Lam CWK, Wu AKL, Ip WK, Lee NLS, Chan IHS, et al. Plasma inflammatory cytokines and chemokines in severe acute respiratory syndrome. *Clin Exp Immunol.* (2004) 136:95–103. doi: 10.1111/j.1365-2249.2004.02415.x
88. Chien J-Y, Hsueh P-R, Cheng W-C, Yu C-J, Yang P-C. Temporal changes in cytokine/chemokine profiles and pulmonary involvement in severe acute respiratory syndrome. *Respirology.* (2006) 11:715–22. doi: 10.1111/j.1440-1843.2006.00942.x
89. Lau SKP, Lau CCY, Chan KH, Li CPY, Chen H, Jin DY, et al. Delayed induction of proinflammatory cytokines and suppression of innate antiviral response by the novel Middle East respiratory syndrome coronavirus: implications for pathogenesis and treatment. *J Gen Virol.* (2013) 94:2679–90. doi: 10.1099/vir.0.055533-0
90. Zhou J, Chu H, Li C, Wong BHY, Cheng ZS, Poon VKM, et al. Active replication of middle east respiratory syndrome coronavirus and aberrant induction of inflammatory cytokines and chemokines in human macrophages: implications for pathogenesis. *J Infect Dis.* (2014) 209:1331–42. doi: 10.1093/infdis/jit504
91. Channappanavar R, Fehr AR, Zheng J, Wohlford-Lenane C, Abrahante JE, Mack M, et al. IFN-I response timing relative to virus replication determines MERS coronavirus infection outcomes. *J Clin Invest.* (2019) 129:3625–39. doi: 10.1172/JCI126363
92. Ng DL, Al Hosani F, Keating MK, Gerber SI, Jones TL, Metcalfe MG, et al. Clinicopathologic, immunohistochemical, and ultrastructural findings of a fatal case of middle east respiratory syndrome coronavirus infection in the United Arab Emirates, April 2014. *Am J Pathol.* (2016) 186:652–8. doi: 10.1016/j.ajpath.2015.10.024
93. Kong SL, Chui P, Lim B, Salto-Tellez M. Elucidating the molecular physiopathology of acute respiratory distress syndrome in severe acute respiratory syndrome patients. *Virus Res.* (2009) 145:260–9. doi: 10.1016/j.virusres.2009.07.014
94. Diao B, Wang C, Tan Y, Chen X, Liu Y, Ning L, et al. Reduction and functional exhaustion of T cells in patients with coronavirus disease 2019 (COVID-19). *Front Immunol.* (2020) 2019:1–14. doi: 10.3389/fimmu.2020.00827
95. Zhe Xu, Lei Shi, Yijin Wang, Jiyuan Zhang, Lei Huang, Chao Zhang, et al. Pathological findings of COVID-19 associated with acute respiratory distress syndrome. *Lancet Respir Med.* (2020) 8:420–2. doi: 10.1016/S2213-2600(20)30076-X
96. Mehta P, Mcauley DF, Brown M, Sanchez E, Tattersall RS, Manson JJ. COVID-19 : consider cytokine storm syndromes and immunosuppression. *Lancet.* (2020) 6736:19–20. doi: 10.1016/S0140-6736(20)30628-0
97. Zhou Y, Fu B, Zheng X, Wang D, Zhao C, Qi Y, et al. Pathogenic T cells and inflammatory monocytes incite inflammatory storm in

severe COVID-19 patients. *Natl Sci Rev.* (2020) 7:1–5. doi: 10.1093/nsr/nwaa041
98. Wen Zhang, Yan Zhao, Fengchun Zhang, Qian Wang, Taisheng Li, Zhengyin Liu, et al. Anti-inflammation treatment of severe coronavirus disease 2019 (COVID-19): from the perspective of clinical immunologists from China. *Clin Immunol.* (2020) 214:108393. doi: 10.1016/j.clim.2020.108393
99. Coperchini F, Chiovato L, Croce L, Magri F, Rotondi M. The cytokine storm in COVID-19: An overview of the involvement of the chemokine/chemokine-receptor system. *Cytokine Growth Factor Rev.* (2020) 53:1–8. doi: 10.1016/j.cytogfr.2020.05.003
100. Pain CE, Felsenstein S, Cleary G, Mayell S, Conrad K, Harave S, et al. Novel paediatric presentation of COVID-19 with ARDS and cytokine storm syndrome without respiratory symptoms. *Lancet Rheumatol.* (2020) 2:19–21. doi: 10.1016/s2665-9913(20)30137-5
101. Xiong Y, Liu Y, Cao L, Wang D, Guo M, Jiang A, et al. Transcriptomic characteristics of bronchoalveolar lavage fluid and peripheral blood mononuclear cells in COVID-19 patients. *Emerg Microbes Infect.* (2020) 9:761–70. doi: 10.1080/22221751.2020.1747363
102. Hertzog PJ, O'Neill LA, Hamilton JA. The interferon in TLR signaling: more than just antiviral. *Trends Immunol.* (2003) 24:534–9. doi: 10.1016/j.it.2003.08.006
103. Wong RSM, Wu A, To KF, Lee N, Lam CWK, Wong CK, et al. Haematological manifestations in patients with severe acute respiratory syndrome:retrospective analysis. (2003) 326:1358–62. doi: 10.1136/bmj.326.7403.1358
104. Li T, Qiu Z, Zhang L, Han Y, He W, Liu Z, et al. Significant changes of peripheral T lymphocyte subsets in patients with severe acute respiratory syndrome. *J Infect Dis.* (2004) 189:4–7. doi: 10.1086/381535
105. Zaki AM, van Boheemen S, Bestebroer TM, Osterhaus ADME, Fouchier RAM. Isolation of a novel coronavirus from a man with pneumonia in Saudi Arabia. *N Engl J Med.* (2012) 367:1814–20. doi: 10.1056/NEJMoa1211721
106. Yang Y, Zhang L, Geng H, Deng Y, Huang B, Guo Y, et al. The structural and accessory proteins M, ORF 4a, ORF 4b, and ORF 5 of Middle East respiratory syndrome coronavirus (MERS-CoV) are potent interferon antagonists. *Protein Cell.* (2013) 4:951–61. doi: 10.1007/s13238-013-3096-8
107. Signaling N-B, Frieman M, Ratia K, Johnston RE, Mesecar AD, Baric RS. Severe acute respiratory syndrome coronavirus papain-like protease ubiquitin-like domain and catalytic domain regulate antagonism of. *J Virol.* (2009) 83:6689–705. doi: 10.1128/JVI.02220-08
108. Devaraj SG, Wang N, Chen Z, Chen Z, Tseng M, Barretto N, et al. Regulation of IRF-3-dependent innate immunity by the papain-like protease domain of the severe acute respiratory syndrome coronavirus. *J Biol Chem.* (2007) 282:32208–21. doi: 10.1074/jbc.M704870200
109. Freundt EC, Yu L, Park E, Lenardo MJ, Xu X-N. Molecular determinants for subcellular localization of the severe acute respiratory syndrome coronavirus open reading frame 3b protein. *J Virol.* (2009) 83:6631–40. doi: 10.1128/jvi.00367-09
110. Kopecky-Bromberg SA, Martinez-Sobrido L, Frieman M, Baric RA, Palese P. Severe acute respiratory syndrome coronavirus open reading frame (ORF) 3b, ORF 6, and nucleocapsid proteins function as interferon antagonists. *J Virol.* (2007) 81:548–57. doi: 10.1128/jvi.01782-06
111. Menachery VD, Eisfeld AJ, Schäfer A, Josset L, Sims AC, Proll S, et al. Pathogenic influenza viruses and coronaviruses utilize similar and contrasting approaches to control interferon-stimulated gene responses. *MBio.* (2014) 5:1–11. doi: 10.1128/mBio.01174-14
112. Blanco-melo D, Nilsson-payant BE, Liu W, Uhl S, Hoagland D, Møller R, et al. Imbalanced host response to SARS-CoV-2 drives development of COVID-19. *Cell.* (2020) 181:1036–45. e1-e9. doi: 10.1016/j.cell.2020.04.026
113. Zheng H, Zhang M, Yang C-X, Zhang N, Wang X-C, Yang X-P, et al. Elevated exhaustion levels and reduced functional diversity of T cells in peripheral blood may predict severe progression in COVID-19 patients. *Cell Mol Immunol.* (2020) 17:541–3. doi: 10.1038/s41423-020-0401-3
114. Qin C, Zhou L, Hu Z, Zhang S, Yang S, Tao Y, et al. Dysregulation of immune response in patients with COVID-19 in Wuhan, China. *Clin Infect Dis.* (2020) 71:762–8. doi: 10.1093/cid/ciaa248
115. Chiappelli F, Khakshooy A, Greenberg G. CoViD-19 immunopathology & immunotherapy. *Bioinformation.* (2020) 16:219–22. doi: 10.6026/97320630016222
116. Stockinger B, Bourgeois C, Kassiotis G. CD4+ memory T cells: functional differentiation and homeostasis. *Immunol Rev.* (2006) 211:39–48. doi: 10.1111/j.0105-2896.2006.00381.x
117. Lees JR, Farber DL. Generation, persistence and plasticity of CD4 T-cell memories. *Immunology.* (2010) 130:463–70. doi: 10.1111/j.1365-2567.2010.03288.x
118. Fan YY, Huang ZT, Li L, Wu MH, Yu T, Koup RA, et al. Characterization of SARS-CoV-specific memory T cells from recovered individuals 4 years after infection. *Arch Virol.* (2009) 154:1093–9. doi: 10.1007/s00705-009-0409-6
119. Tang F, Quan Y, Xin Z-T, Wrammert J, Ma M-J, Lv H, et al. Lack of peripheral memory B cell responses in recovered patients with severe acute respiratory syndrome: a six-year follow-up study. *J Immunol.* (2011) 186:7264–8. doi: 10.4049/jimmunol.0903490
120. Peng H, Yang L, Wang L, Li J, Huang J, Lu Z, et al. Long-lived memory T lymphocyte responses against SARS coronavirus nucleocapsid protein in SARS-recovered patients. *Virology.* (2006) 351:466–75. doi: 10.1016/j.virol.2006.03.036
121. Zhao J, Zhao J, Mangalam AK, Channappanavar R, Fett C, Meyerholz DK, et al. Airway memory CD4+ T cells mediate protective immunity against emerging respiratory coronaviruses. *Immunity.* (2016) 44:1379–91. doi: 10.1016/j.immuni.2016.05.006
122. Sun S, Chen Q, Gu H, Yang G, Wang Y, Huang X, et al. A mouse model of SARS-CoV-2 infection and pathogenesis. *Cell Host Microbe.* (2020) 28:124–33.e4. doi: 10.1016/j.chom.2020.05.020
123. Zumla A, Chan JFW, Azhar EI, Hui DSC, Yuen KY. Coronaviruses-drug discovery and therapeutic options. *Nat Rev Drug Discov.* (2016) 15:327–47. doi: 10.1038/nrd.2015.37
124. Jin YH, Cai L, Cheng ZS, Cheng H, Deng T, Fan YP, et al. A rapid advice guideline for the diagnosis and treatment of 2019 novel coronavirus (2019-nCoV) infected pneumonia (standard version). *Mil Med Res.* (2020) 7:1–23. doi: 10.1186/s40779-020-0233-6
125. Zhu Z, Chakraborti S, He Y, Roberts A, Sheahan T, Xiao D, et al. Potent cross-reactive neutralization of SARS coronavirus isolates by human monoclonal antibodies. *Proc Natl Acad Sci USA.* (2007) 104:12123–8. doi: 10.1073/pnas.0701000104
126. Jiang L, Wang N, Zuo T, Shi X, Poon KMV, Wu Y, et al. Potent neutralization of MERS-CoV by human neutralizing monoclonal antibodies to the viral spike glycoprotein. *Sci Transl Med.* (2014) 6:234ra59. doi: 10.1126/scitranslmed.3008140
127. Zhang C, Wu Z, Li J-W, Zhao H, Wang G-Q. The cytokine release syndrome (CRS) of severe COVID-19 and Interleukin-6 receptor (IL-6R) antagonist Tocilizumab may be the key to reduce the mortality. *Int J Antimicrob Agents.* (2020) 55:105954. doi: 10.1016/j.ijantimicag.2020.105954
128. Monteleone G, Sarzi-Puttini PC, Ardizzone S. Preventing COVID-19-induced pneumonia with anticytokine therapy. *The Lancet Rheumatogy.* (2020) 2:e255–6. doi: 10.1016/S2665-9913(20)30092-8
129. FDA Approves Emergency IND Use of Humanigen's Lenzilumab For Compassionate Use In COVID-19 Patients. Available online at: https://apnews.com/ACCESSWIRE/738d3526740dee777dacee2b6b8a836f (accessed April 9, 2020).
130. Altasciences. Altasciences Completes Phase I Study on Gimsilumab for ARDS in COVID-19. (2020). Available online at: https://www.altasciences.com/press-release/altasciences-completes-phase-i-study-gimsilumab-ards-covid-19 (accessed April 9, 2020).
131. Sallard E, Lescure FX, Yazdanpanah Y, Mentre F, Peiffer-Smadja N. Type 1 interferons as a potential treatment against COVID-19. *Antiviral Res.* (2020) 178:1–4. doi: 10.1016/j.antiviral.2020.104791
132. Prokunina-Olsson L, Alphonse N, Dickenson RE, Durbin JE, Glenn JS, Hartmann R, et al. COVID-19 and emerging viral infections: the case for interferon lambda. *J Exp Med.* (2020) 217:e20200653. doi: 10.1084/jem.20200653
133. Morse JS, Lalonde T, Xu S, Liu WR. Learning from the past: possible urgent prevention and treatment options for severe acute respiratory infections caused by 2019-nCoV. *ChemBioChem.* (2020) 21:730–8. doi: 10.1002/cbic.202000047
134. Liu C, Zhou Q, Li Y, Garner LV, Watkins SP, Carter LJ, et al. Research and development on therapeutic agents and vaccines for COVID-19

and related human coronavirus diseases. *ACS Cent Sci.* (2020) 6:315–31. doi: 10.1021/acscentsci.0c00272
135. Ortiz-Alcantara J, Bhardwaj K, Palaninathan S, Frieman M, Baric RS, Kao CC. Small molecule inhibitors of the SARS-CoV Nsp15 endoribonuclease. *Virus Adapt Treat.* (2010) 2:125–33. doi: 10.2147/VAAT.S12733
136. Sheahan TP, Sims AC, Zhou S, Graham RL, Pruijssers AJ, Agostini ML, et al. An orally bioavailable broad-spectrum antiviral inhibits SARS-CoV-2 in human airway epithelial cell cultures and multiple coronaviruses in mice. *Sci Transl Med.* (2020) 5883:1–21. doi: 10.1126/scitranslmed.abb5883
137. Richardson P, Griffin I, Tucker C, Smith D, Oechsle O, Phelan A, et al. Baricitinib as potential treatment for 2019-nCoV acute respiratory disease. *Lancet.* (2020) 395:e30–e31. doi: 10.1016/S0140-6736(20)30304-4
138. Sheahan TP, Sims AC, Leist SR, Schäfer A, Won J, Brown AJ, et al. Comparative therapeutic efficacy of remdesivir and combination lopinavir, ritonavir, and interferon beta against MERS-CoV. *Nat Commun.* (2020) 11:1–14. doi: 10.1038/s41467-019-13940-6
139. Cao B, Wang Y, Wen D, Liu W, Wang J, Fan G, et al. A trial of lopinavir–ritonavir in adults hospitalized with severe covid-19. *N Engl J Med.* (2020) 382:1787–99. doi: 10.1056/nejmoa2001282
140. Guo D. Old weapon for new enemy: drug repurposing for treatment of newly emerging viral diseases. *Virol Sin.* (2020) 35:12250. doi: 10.1007/s12250-020-00204-7
141. Wang M, Cao R, Zhang L, Yang X, Liu J, Xu M, et al. Remdesivir and chloroquine effectively inhibit the recently emerged novel coronavirus (2019-nCoV) in vitro. *Cell Res.* (2020) 30:269–71. doi: 10.1038/s41422-020-0282-0
142. Omrani AS, Saad MM, Baig K, Bahloul A, Abdul-Matin M, Alaidaroos AY, et al. Ribavirin and interferon alfa-2a for severe Middle East respiratory syndrome coronavirus infection: a retrospective cohort study. *Lancet Infect Dis.* (2014) 14:1090–5. doi: 10.1016/S1473-3099(14)70920-X
143. Warren TK, Wells J, Panchal RG, Stuthman KS, Garza NL, Van Tongeren SA, et al. Protection against filovirus diseases by a novel broad-spectrum nucleoside analogue BCX4430. *Nature.* (2014) 508:402–5. doi: 10.1038/nature13027
144. Devaux CA, Rolain JM, Colson P, Raoult D. New insights on the antiviral effects of chloroquine against coronavirus: what to expect for COVID-19? *Int J Antimicrob Agents.* (2020) 55:105938. doi: 10.1016/j.ijantimicag.2020.105938
145. Schrezenmeier E, Dörner T. Mechanisms of action of hydroxychloroquine and chloroquine: implications for rheumatology. *Nat Rev Rheumatol.* (2020) 16:155–66. doi: 10.1038/s41584-020-0372-x
146. ClinicalTrials.gov. The Efficacy of Lopinavir Plus Ritonavir and Arbidol Against Novel Coronavirus Infection. (2020). Available online at: https://clinicaltrials.gov/ct2/show/NCT04252885 (accessed April 10, 2020).
147. Groneberg DA, Hilgenfeld R, Zabel P. Molecular mechanisms of severe acute respiratory syndrome (SARS). *Respir Res.* (2005) 6:1–16. doi: 10.1186/1465-9921-6-8
148. Wan Y, Shang J, Graham R, Baric RS, Li F. Receptor recognition by the novel coronavirus from Wuhan: an analysis based on decade-long structural studies of SARS coronavirus. *J Virol.* (2020) 94:1–9. doi: 10.1128/jvi.00127-20
149. Lo MK, Jordan R, Arvey A, Sudhamsu J, Shrivastava-Ranjan P, Hotard AL, et al. GS-5734 and its parent nucleoside analog inhibit Filo-, Pneumo-, and Paramyxoviruses. *Sci Rep.* (2017) 7:1–7. doi: 10.1038/srep43395
150. de Wit E, Feldmann F, Cronin J, Jordan R, Okumura A, Thomas T, et al. Prophylactic and therapeutic remdesivir (GS-5734) treatment in the rhesus macaque model of MERS-CoV infection. *Proc Natl Acad Sci USA.* (2020) 117:6771–76. doi: 10.1073/pnas.1922083117
151. Lo MK, Feldmann F, Gary JM, Jordan R, Bannister R, Cronin J, et al. Remdesivir (GS-5734) protects African green monkeys from Nipah virus challenge. *Sci Transl Med.* (2019) 11:1–7. doi: 10.1126/scitranslmed.aau9242
152. Holshue ML, DeBolt C, Lindquist S, Lofy KH, Wiesman J, Bruce H, et al. First case of 2019 novel coronavirus in the United States. *N Engl J Med.* (2020) 382:929–36. doi: 10.1056/NEJMoa2001191
153. Beigel JH, Tomashek KM, Dodd LE, Mehta AK, Zingman BS, Kalil AC, et al. Remdesivir for the Treatment of Covid-19 - Preliminary Report. *N Engl J Med.* (2020) NEJMoa2007764. doi: 10.1056/NEJMoa2007764
154. De Clercq E. New nucleoside analogues for the treatment of hemorrhagic fever virus infections. *Chem - An Asian J.* (2019) 14:3962–8. doi: 10.1002/asia.201900841
155. Aguiar ACC, Murce E, Cortopassi WA, Pimentel AS, Almeida MMFS, Barros DCS, et al. Chloroquine analogs as antimalarial candidates with potent in vitro and in vivo activity. *Int J Parasitol Drugs Drug Resist.* (2018) 8:459–64. doi: 10.1016/j.ijpddr.2018.10.002
156. Vincent MJ, Bergeron E, Benjannet S, Erickson BR, Rollin PE, Ksiazek TG, et al. Chloroquine is a potent inhibitor of SARS coronavirus infection and spread. *Virol J.* (2005) 2:1–10. doi: 10.1186/1743-422X-2-69
157. Savarino A, Boelaert JR, Cassone A, Majori G, Cauda R. Effects of chloroquine on viral infections: an old drug against today's diseases? *Lancet Infect Dis.* (2003) 3:722–7. doi: 10.1016/S1473-3099(03)00806-5
158. Boulware DR, Pullen MF, Bangdiwala AS, Pastick KA, Lofgren SM, Okafor EC, et al. A randomized trial of hydroxychloroquine as postexposure prophylaxis for covid-19. *N Engl J Med.* (2020) 1–9. doi: 10.1056/NEJMoa2016638
159. Arabi YM, Alothman A, Balkhy HH, Al-Dawood A, AlJohani S, Al Harbi S, et al. Treatment of Middle East Respiratory Syndrome with a combination of lopinavir-ritonavir and interferon-β1b (MIRACLE trial): study protocol for a randomized controlled trial. *Trials.* (2018) 19:1–13. doi: 10.1186/s13063-017-2427-0
160. Chen L, Gui C, Luo X, Yang Q, Gunther S, Scandella E, et al. Cinanserin is an inhibitor of the 3c-like proteinase of severe acute respiratory syndrome coronavirus and strongly reduces virus replication in vitro. *J Virol.* (2005) 79:7095–103. doi: 10.1128/jvi.79.11.7095-7103.2005
161. Jo S, Kim S, Shin DH, Kim MS. Inhibition of SARS-CoV 3CL protease by flavonoids. *J Enzyme Inhib Med Chem.* (2020) 35:145–51. doi: 10.1080/14756366.2019.1690480
162. Park JY, Jeong HJ, Kim JH, Kim YM, Park SJ, Kim D, et al. Diarylheptanoids from Alnus japonica inhibit papain-like protease of severe acute respiratory syndrome coronavirus. *Biol Pharm Bull.* (2012) 35:2036–42. doi: 10.1248/bpb.b12-00623
163. Zhou Y, Hou Y, Shen J, Huang Y, Martin W, Cheng F. Network-based drug repurposing for novel coronavirus 2019-nCoV/SARS-CoV-2. *Cell Discov.* (2020) 6:14. doi: 10.1038/s41421-020-0153-3
164. Arabi YM, Hajeer AH, Luke T, Raviprakash K, Balkhy H, Johani S, et al. Feasibility of using convalescent plasma immunotherapy for MERS-CoV infection, Saudi Arabia. *Emerg Infect Dis.* (2016) 22:1554–61. doi: 10.3201/eid2209.151164
165. Zhou B, Zhong N, Guan Y. Treatment with convalescent plasma for influenza A (H5N1) infection. *N Engl J Med.* (2007) 357:1450–1. doi: 10.1056/NEJMc070359
166. Casadevall A, Pirofski L. The convalescent sera option for containing COVID-19. *J Clin Invest.* (2020) 130:1545–48. doi: 10.1172/jci138003
167. Thevarajan I, Nguyen THO, Koutsakos M, Druce J, Caly L, van de Sandt CE, et al. Breadth of concomitant immune responses prior to patient recovery: a case report of non-severe COVID-19. *Nat Med.* (2020) 26:453–5. doi: 10.1038/s41591-020-0819-2
168. Duan K, Liu B, Li C, Zhang H, Yu T, Qu J, et al. Effectiveness of convalescent plasma therapy in severe COVID-19 patients. *Proc Natl Acad Sci USA.* (2020) 117:202004168. doi: 10.1073/pnas.2004168117
169. Cheng Y, Wong R, Soo YOY, Wong WS, Lee CK, Ng MHL, et al. Use of convalescent plasma therapy in SARS patients in Hong Kong. *Eur J Clin Microbiol Infect Dis.* (2005) 24:44–6. doi: 10.1007/s10096-004-1271-9
170. FDA. Recommendations for Investigational COVID-19 Convalescent Plasma. (2020). Available online at: https://www.fda.gov/vaccines-blood-biologics/investigational-new-drug-ind-or-device-exemption-ide-process-cber/recommendations-investigational-covid-19-convalescent-plasma# Patient Eligibility (accessed April 9, 2020).
171. Roper RL, Rehm KE. SARS vaccines: where are we? *Expert Rev Vaccines.* (2009) 8:887–98. doi: 10.1586/erv.09.43
172. Ishii K, Hasegawa H, Nagata N, Ami Y, Fukushi S, Taguchi F, et al. Neutralizing antibody against severe acute respiratory syndrome (SARS)-coronavirus spike is highly effective for the protection of mice in the murine SARS model. *Microbiol Immunol.* (2009) 53:75–82. doi: 10.1111/j.1348-0421.2008.00097.x

173. Guo JP, Petric M, Campbell W, McGeer PL. SARS corona virus peptides recognized by antibodies in the sera of convalescent cases. *Virology*. (2004) 324:251–6. doi: 10.1016/j.virol.2004.04.017
174. Tsunetsugu-Yokota Y, Ohnishi K, Takemori T. Severe acute respiratory syndrome (SARS) coronavirus: application of monoclonal antibodies and development of an effective vaccine. *Rev Med Virol.* (2006) 16:117–31. doi: 10.1002/rmv.492
175. Wang N, Shang J, Jiang S, Du L. Subunit vaccines against emerging pathogenic human coronaviruses. *Front Microbiol.* (2020) 11:289. doi: 10.3389/fmicb.2020.00298
176. Gao Q, Bao L, Mao H, Wang L, Xu K, Yang M, et al. Development of an inactivated vaccine candidate for SARS-CoV-2. *Science.* (2020) 369:77–81. doi: 10.1126/science.abc1932
177. van Doremalen N, Lambe T, Spencer A, Belij-Rammerstorfer S, Purushotham JN, Port JR, et al. ChAdOx1 nCoV-19 vaccination prevents SARS-CoV-2 pneumonia in rhesus macaques. *bioRxiv*. (2020) 2020.05.13.093195. doi: 10.1101/2020.05.13.093195
178. Zhu F-C, Li Y-H, Guan X-H, Hou L-H, Wang W-J, Li J-X, et al. Safety, tolerability, and immunogenicity of a recombinant adenovirus type-5 vectored COVID-19 vaccine: a dose-escalation, open-label, non-randomised, first-in-human trial. *Lancet.* (2020) 6736:1–10. doi: 10.1016/S0140-6736(20)31208-3
179. Bolles M, Deming D, Long K, Agnihothram S, Whitmore A, Ferris M, et al. A double-inactivated severe acute respiratory syndrome coronavirus vaccine provides incomplete protection in mice and induces increased eosinophilic proinflammatory pulmonary response upon challenge. *J Virol.* (2011) 85:12201–15. doi: 10.1128/jvi.06048-11
180. Tseng C Te, Sbrana E, Iwata-Yoshikawa N, Newman PC, Garron T, Atmar RL, et al. Immunization with SARS coronavirus vaccines leads to pulmonary immunopathology on challenge with the SARS virus. *PLoS ONE.* (2012) 7:e35421. doi: 10.1371/journal.pone.0035421
181. Iwata-Yoshikawa N, Uda A, Suzuki T, Tsunetsugu-Yokota Y, Sato Y, Morikawa S, et al. Effects of toll-like receptor stimulation on eosinophilic infiltration in lungs of BALB/c mice immunized with UV-inactivated severe acute respiratory syndrome-related coronavirus vaccine. *J Virol.* (2014) 2:e255–6. doi: 10.1128/jvi.00983-14
182. Jiang S, Hillyer C, Du L. Neutralizing antibodies against SARS-CoV-2 and other human coronaviruses. *Trends Immunol.* (2020) 41: 355–9. doi: 10.1016/j.it.2020.03.007
183. Tian X, Li C, Huang A, Xia S, Lu S, Shi Z, et al. Potent binding of 2019 novel coronavirus spike protein by a SARS coronavirus-specific human monoclonal antibody. *Emerg Microbes Infect.* (2020) 9:382–5. doi: 10.1080/22221751.2020.1729069
184. Ni L, Ye F, Cheng M, Qin C, Chen F, Ni L, et al. Detection of SARS-CoV-2-specific humoral and cellular immunity in COVID-19 convalescent individuals. *Immunity*. (2020) 52:1–7. doi: 10.1016/j.immuni.2020.04.023
185. Wang C, Li W, Drabek D, Okba NMA, Haperen R Van, Osterhaus ADME, et al. A human monoclonal antibody blocking SARS-CoV-2 infection. *Nat Commun.* (2020) 11:1–6. doi: 10.1038/s41467-020-16256-y
186. Wu Y, Li C, Xia S, Xiaolong T, Kong Y, Wang Z, et al. Identification of human single-domain antibodies against SARS-CoV-2. *Cell Host Microbe.* (2020) 27:1–8. doi: 10.1016/j.chom.2020.04.023
187. Kim E, Erdos G, Huang S, Kenniston TW, Balmert SC, Donahue Carey C, et al. Microneedle array delivered recombinant coronavirus vaccines: immunogenicity and rapid translational development. *EBioMedicine.* (2020) 55:102743. doi: 10.1016/j.ebiom.2020.102743
188. Amanat Fatima KF. SARS-CoV-2 vaccines: status report. *Cell.* (2020) 52:1–7. doi: 10.1016/j.immuni.2020.03.007
189. Conti P, Ronconi G, Caraffa A, Gallenga CE, Ross R, Frydas I, et al. Induction of pro-inflammatory cytokines (IL-1 and IL-6) and lung inflammation by Coronavirus-19 (COVI-19 or SARS-CoV-2): anti-inflammatory strategies. *J Biol Regul Homeost Agents.* (2020). doi: 10.23812/CONTI-E. [Epub ahead of print].
190. Kritas SK, Ronconi G, Caraffa A, Gallenga CE, Ross R, Conti P. Mast cells contribute to coronavirus-induced inflammation: new anti-inflammatory strategy. *J Biol Regul Homeost Agents.* (2020). doi: 10.23812/20-Editorial-Kritas. [Epub ahead of print].
191. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet.* (2020) 395:497–506. doi: 10.1016/S0140-6736(20)30183-5
192. Kurtzberg J, Abdel-Azim H, Carpenter P, Chaudhury S, Horn B, Mahadeo K, et al. A phase 3, single-arm, prospective study of remestemcel-l, ex vivo culture-expanded adult human mesenchymal stromal cells for the treatment of pediatric patients who failed to respond to steroid treatment for acute graft-versus-host disease. *Biol Blood Marrow Transplant.* (2020) 26:845–54. doi: 10.1016/j.bbmt.2020.01.018
193. Mannon PJ. Remestemcel-L: Human mesenchymal stem cells as an emerging therapy for Crohn's disease. *Expert Opin Biol Ther.* (2011) 11:1249–56. doi: 10.1517/14712598.2011.602967
194. Leng Z, Zhu R, Hou W, Feng Y, Yang Y, Han Q, et al. Transplantation of ACE2- mesenchymal stem cells improves the outcome of patients with COVID-19 pneumonia. *Aging Dis.* (2020) 11:216. doi: 10.14336/AD.2020.0228
195. Mesoblast Limited. *Mesoblast To Evaluate Anti-Inflammatory Cell Therapy Remestemcel-L for Treatment of Covid-19 Lung Disease*. Melbourne; New York, NY: ASX announcement (2020). p. 2060–2.
196. Fang L, Karakiulakis G, Roth M. Are patients with hypertension and diabetes mellitus at increased risk for COVID-19 infection? *Lancet Respir.* (2020) 8:e21. doi: 10.1016/S2213-2600(20)30116-8

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pediatrics

REVIEW
published: 07 August 2020
doi: 10.3389/fped.2020.00511

Potential Effect of COVID-19 on Maternal and Infant Outcome: Lesson From SARS

*Yun Wang[1], Yiliang Wang[2], Xiaoxue Han[1], Jiazhuo Ye[1] and Ruiman Li[1]**

[1] Department of Obstetrics and Gynecology, The First Affiliated Hospital of Jinan University, Guangzhou, China, [2] Guangzhou Jinan Biomedicine Research and Development Center, College of Life Science and Technology, Jinan University, Guangzhou, China

The coronavirus disease 2019 (COVID-19), caused by SARS-CoV-2, is highly infectious and its ongoing outbreak has been declared a global pandemic by the WHO. Pregnant women are susceptible to respiratory pathogens and the development of severe pneumonia, suggesting the urgent need to assess the potential maternal and infant outcome of pregnancy with COVID-19. The intrauterine vertical transmission potential of SARS-CoV-2 also remains controversial. Herein, we discuss the potential effect of COVID-19 on maternal and infant outcomes based on current studies, including those published in Chinese, in a total of 80 mothers with COVID-19 and 80 infants. We also comprehensively explored the mother-to-child transmission routes of SARS-CoV-2, in particular the route of intrauterine vertical transmission. Given SARS-CoV-2 is a sister to SARS-CoV, of the SARS-related coronavirus species, we made a comprehensive comparison between them to learn from experiences with SARS. Although there is no evidence supporting the intrauterine vertical transmission of SARS-CoV-2, our comprehensive analysis suggests that the adverse maternal and infant outcomes caused by COVID-19 cannot be underestimated. Further, we speculated that the inconsistency between nucleic acids and serological characteristics IgM to SARS-CoV-2 of infants' specimens may be caused by the disruption of the amniotic barrier by the inflammatory factors induced by SARS-CoV-2 infection. Our review is beneficial to understand the effect of SARS-CoV-2 on maternal and infant outcomes.

Keywords: COVID-19, SARS-CoV-2, maternal outcome, infant outcome, SARS-CoV

OPEN ACCESS

Edited by:
Marzia Duse,
Sapienza University of Rome, Italy

Reviewed by:
Arturo Solis-Moya,
Dr. Carlos Sáenz Herrera National Children's Hospital, Costa Rica
Antonio Arbo,
Institute of Tropical Medicine, Paraguay

****Correspondence:***
Ruiman Li
ruiman_li@126.com

Specialty section:
This article was submitted to Pediatric Infectious Diseases, a section of the journal Frontiers in Pediatrics

Received: *17 April 2020*
Accepted: *20 July 2020*
Published: *07 August 2020*

Citation:
Wang Y, Wang Y, Han X, Ye J and Li R (2020) Potential Effect of COVID-19 on Maternal and Infant Outcome: Lesson From SARS. Front. Pediatr. 8:511. doi: 10.3389/fped.2020.00511

INTRODUCTION

At the beginning of December 2019, a cluster of pneumonia cases with unknown causes were reported (1, 2). A subsequent high-throughput sequencing revealed that the pneumonia epidemic resulted from a novel beta coronavirus tentatively named "2019 novel coronavirus" (2019-nCoV) that was subsequently termed "severe acute respiratory syndrome coronavirus 2" (SARS-CoV-2) (3–5). SARS-CoV-2 is a sister to SARS-CoV, of the SARS-related coronavirus species (4–8). Pneumonia caused by SARS-CoV-2 was correspondingly termed "coronavirus disease 2019" (COVID-19) (4, 5). The ongoing outbreak of COVID-19 has posed a great threat to global human health, which has been declared by the WHO as a global public health emergency (1, 2). As shown by the Center for Systems Science and Engineering at Johns Hopkins University (last updated on 07/07/2020), the global cumulative

number of confirmed cases has reached 11,779,263, with 6,758,547 cures, and 540,948 deaths (9). Due to the high transmissibility of COVID-19, the prevention, and control of COVID-19 infection has become a major concern (4, 5). Pregnant women are more susceptible to respiratory pathogens and the development of severe pneumonia than the general population, especially so for those with chronic diseases or maternal complications (4). The physiologic changes in pregnancy, including altered cell-mediated immunity, and alterations in pulmonary function, may confer the susceptibility and severity of pneumonia to pregnant women (6, 10, 11). Pneumonia arising from infectious etiology is the most common non-obstetric infectious condition that occurs in pregnant women (6, 12, 13). In particular, universal SARS-Cov-2 screening for women admitted for delivery found that all women with positive test results were asymptomatic at the time of testing (14, 15). Therefore, the effect of SARS-CoV-2 infection on maternal and infant outcomes needs to be explored, especially the intrauterine vertical transmission potential of COVID-19. Moreover, in the use of a reverse transcriptase-polymerase chain reaction (RT-PCR) and the specific antibody to SARS-CoV-2 of neonate samples remains controversial (4, 5, 16–20). Given SARS-CoV-2 is a sister of SARS-CoV, it is important for us to learn from the experience of preventing and controlling SARS-CoV among pregnant people. In this review, we made a comprehensive comparison of SARS-CoV-2 and SARS-CoV in genetic, infection, transmission, and clinical characteristics. Based on such a comparison, we summarized the potential maternal, and infant outcomes from pregnancy with COVID-19 or SARS. Further, we discuss the potential of mother-to-child transmission of SARS-CoV-2, in particular the possibility of intrauterine vertical transmission. The guidelines for those women with SARS-CoV-2 infections during pregnancy and puerperium prepared by numerous experts were also briefly presented (21). Considering the ongoing global public health emergency, we believe that our review is important for understanding the mother-to-child transmission potential of SARS-CoV-2 and its implication for the safe management of COVID-19 in pregnancy.

COMPARISON BETWEEN SARS-CoV AND SARS-CoV-2

The pathogen contributing to the COVID-19 epidemic was tentatively named "2019-nCoV" (3, 22). Based on phylogeny, taxonomy, and established practice, the Coronavirus Study Group (CSG) of the International Committee on Taxonomy of Viruses formally recognizes this virus as a sister to SARS-CoV and designates it as SARS-CoV-2 (8). A coronavirus is spherical, enveloped, and the largest of the positive-strand RNA virus and SARS-CoV-2 is the seventh member of enveloped RNA coronaviruses with the ability to infect humans (2, 6). The coronaviruses currently known to infect humans include HCoV-229E and HCoV-NL63 (*Alphacoronavirus* genus), HCoV-OC43, HCoV-HKU1, MERS-CoV, SARS-CoV, and SARS-CoV-2 (*Betacoronavirus* genus) (23). SARS-CoV is the pathogen that caused the SARS epidemic from 2002 to 2003 (24). There were 8,422 cases and 916 deaths in 29 countries, with most of them having occurred in mainland China by 31 July 2003 (24).

Given the great similarity between SARS-CoV and SARS-CoV-2, a comprehensive comparison between these two viruses can help us learn from the SARS epidemic to control and prevent COVID-19. Their comparisons were mainly presented according to their clinical and viral characteristics (**Table 1**). In general, there was a 79.5% similarity in the whole genome between SARS-CoV and SARS-CoV-2, while only a 74.9% similarity in the gene coding spike glycoprotein (3, 22, 25). Both SARS-CoV-2 and SARS-CoV spread rapidly from human-to-human transmission (7, 28–32). SARS-CoV can be spread via respiratory droplets, secretions, nosocomial contacts, and mechanical aerosols, such as the aerosols arising from the flushing of toilets (33–35). SARS-CoV-2 seems to spread more easily among humans than SARS-CoV, which may result from the various modes of transmission and its high affinity with its receptor angiotensin-converting enzyme 2 (ACE2) (**Table 1**). The latest pilot experiment confirmed that 4 out of 62 stool specimens (6.5%) tested positive to SARS-CoV-2, and another 4 patients who tested positive toward SARS-CoV-2 in rectal swabs also had SARS-CoV-2 detected in their gastrointestinal tract, saliva, or urine (7). The results suggest the possibility of transmission via aerosols arising from the flushing of toilets. In particular, SARS-CoV-2 can be detected in esophageal erosion and bleeding sites in cases with severe peptic ulcers after symptom onset (7). Although these results only suggest the existence of SARS-CoV-2 nucleotides fragments in these samples, Sun et al. (36) reported that urine samples of COVID-19 patients can isolate SARS-CoV-2 with the infectious ability, suggesting the existence of infectious viral particles in these samples. Moreover, the gastrointestinal tract highly expressed ACE2 as indicated by the Human Protein Atlas, which may explain the existence of SARS-CoV-2 in urine and stool specimens (7). Indeed, the 20–30-fold higher affinity of SARS-CoV-2 spike glycoproteins binding to ACE2 than the SARS-CoV spike protein may also enable the rapid transmission of COVID-19 (25–27). Collectively, the mounting routes of transmission and high affinity with ACE2 might jointly contribute to the rapid spread of SARS-CoV-2. However, COVID-19 exhibited a lower-case fatality rate than SARS (7). The median incubation period of SARS-CoV is also longer than SARS-CoV (7).

Despite the high phylogenetic homogeneity between SARS-CoV-2 and SARS-CoV, there are still some clinical characteristics differentiating COVID-19 from SARS. The symptoms of those infected with SARS-CoV have been more common in respiratory out-patient clinics and wards (37, 38). After analyzing the 1,099 COVID-19 patients, Guan et al. (7) found that the typical radiological finding on chest computed tomographies is ground-glass opacity with a ratio of 50.00%. Consistent with previous

Abbreviations: SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SARS-CoV, severe acute respiratory syndrome coronavirus 1; SARS, severe acute respiratory syndrome; COVID-19, 2019 novel coronavirus disease; ACE2, angiotensin-converting enzyme 2; LGA, large-for-gestational-age; SGA, small-for-gestational-age; TTN, transient tachypnea of the newborn; nCPAP, nasal-Continuous Positive Airway Pressure.

TABLE 1 | Comparisons between SARS-CoV and SARS-CoV-2.

Items	SARS-CoV	SARS-CoV-2	References
First location	Guangdong, China	Wuhan, Hubei, China	(7)
Classification	Genus *Betacoronavirus*, subgenus *Sarbecovirus*	Genus *Betacoronavirus*, subgenus *Sarbecovirus*	(3, 22, 25)
Disease name	SARS	COVID-19	(8, 24)
Clinical characteristics	Fever (99–100%); Cough (62–100%); Diarrhea (20–25%); Chest radiograph abnormality (94–100%); Leukopenia (25–35%); Lymphopenia (68–85%); Thrombocytopenia (40–45%)	Fever (83–98%); Cough (76–82%); Diarrhea (2–3%); Chest radiograph abnormality (76.4%); Leukopenia (5–9%); Lymphopenia (35–63%); Thrombocytopenia (5–12%)	(7)
Case fatality rate (%)	11	1.4–2.1	(7, 24)
Basic reproduction number (R_0)	2.0–3.0	2.2–2.68	(24, 26)
Median and range of incubation period (days)	4.6 (2.0–14.0)	5.2 (95%CI: 4.1–7.0)	(7, 24)
Receptor of virus spike protein	ACE2	ACE2(With a 20–30-fold higher affinity than SARS-CoV)	(25–27)
Conventional routes of transmission	Respiratory droplets, secretions, nosocomial contact, and mechanical aerosols	Respiratory droplets, direct contact, fomite transmission	(7, 24)
Evidence of supporting Intrauterine vertical transmission	No	No	(4–6)

publications (1, 32, 39), the most common clinical characteristics of COVID-19 are fever (87.9%) and cough (67.7%), but not the gastrointestinal symptom that is more frequently observed in SARS (7). The absence of fever in COVID-19 seems to be more frequent than in SARS-CoV (1%), as fever occurred in only 43.8% of COVID-19 patients on initial presentation (40), implying the limitation of focusing on fever detection in defining surveillance cases (41).

MATERNAL AND INFANT OUTCOMES FROM SARS-CoV-INFECTED PREGNANT WOMEN

Although there were relatively few cases of patients infected with SARS during pregnancy based on previous clinical studies and case reports, there were more than 100 cases of SARS-CoV infections that occurred in pregnant women as estimated by the WHO (24). SARS-CoV infection during pregnancy was associated with a risk of adverse maternal and neonatal complications, including intrauterine growth restriction, preterm delivery, spontaneous miscarriage, severe maternal illnesses, such as, admission to the intensive care unit (ICU), renal failure, and disseminated intravascular coagulopathy, and death (4, 6, 13, 42–46). In detail, a case-control study found that the clinical characteristics of SARS in pregnant women were similar to those reported for non-pregnant patients with SARS (47). However, all the pregnant women with SARS required endotracheal intubation, and six were admitted to the ICU, whereas the intubation rate and ICU admission rate in the non-pregnant group was only 17.5 and 12.5%, respectively (47). There were three deaths among pregnant women with SARS, while no deaths occurred in the non-pregnant women with SARS-CoV infection. Both renal failure and disseminated intravascular coagulopathy were developed more frequently in pregnant SARS patients than non-pregnant SARS women (47). Zhang et al. (46) also reported SARS-CoV infections in five primigravids while none of the five infants had virologic evidence of SARS-CoV. In a more detailed case report, Robertson et al. (48) described a 36-year-old pregnant woman with a SARS-CoV infection. Obstetrical ultrasounds revealed a low-lying placenta (placenta previa), but the pregnancy was otherwise normal. The cesarean section was performed at 38 weeks gestation due to the placenta previa and a healthy baby girl was delivered (48, 49). Antibodies against SARS-CoV were tested positive from the maternal serum, umbilical cord blood, and breast milk. No viral RNA was detected in specimens of maternal serum and whole blood, or in swabs from the maternal nasopharynx and rectum, post-delivery placenta, umbilical cord blood, amniotic fluid, and breast milk. However, no clinical specimens were available for testing from the infant in this study (48). Another 38-year-old woman was exposed to SARS-CoV in the same hotel as the aforementioned patients (50). The serum samples taken on days 28 and 64 post-onset of illness tested positive for antibodies against SARS-CoV. Her pregnancy continued and was unremarkable except for developing elevated glucose levels. Due to the preterm rupture of membranes and fetal distress, this patient underwent a cesarean section at 36 weeks gestation and obtained a healthy baby boy. The mother's serum samples at the time of delivery were positive for antibodies against SARS-CoV, but both umbilical cord blood and placenta were negative. Also, breast milk sampled 12 and 30 days after delivery were negative for SARS-CoV antibodies. The specimens, including maternal blood, stool, nasopharynx samples, and umbilical cord blood of the infant, were negative for SARS-CoV

RNA. Consistently, the stool samples from the neonate obtained on days-of-life 12 and 30 were negative for SARS-CoV RNA. Yudin et al. (51) reported a 33-year-old pregnant woman who was admitted to the hospital at 31 weeks' gestation due to SARS. Following a 21-day stay in the hospital, the antibody against SARS-CoV tested positive, while she had a normal labor delivery. Together, there were no cases of vertical transmission identified among the pregnant women with SARS-CoV infection (24, 43, 45, 52). However, the effect of SARS on maternal outcomes seems to be associated with the stage of pregnancy when the onset of SARS-CoV occurs (44, 53). Wong et al. (44) found that the SARS-CoV infections present during the first trimester of pregnancy was more likely to cause spontaneous miscarriages, while infections present after 24 weeks of pregnancy developed into delivered preterm.

MATERNAL AND INFANT OUTCOMES FROM PREGNANT WOMEN WITH COVID-19

Current research involving pregnancy with COVID-19 were listed in **Table 2**. Results seems to be inconsistent between antibody-based serological characteristics and RT-PCR-based virologic evidence of infants. Specifically, a retrospective study published in *The Lancet* from (5) reported that the clinical characteristics of SARS-CoV-2 infection in pregnancy were similar to those reported for non-pregnant adults with a SARS-CoV-2 infection. In brief, the typical symptoms, including fever (in seven of nine patients), cough (in four), myalgia (in three), malaise (in two), and sore throat (in two), were observed in these patients, while none of them developed severe COVID-19 pneumonia or died. All patients underwent a cesarean section and their live births had a 1-min Apgar score of 8–9 and a 5-min Apgar score of 9–10 (5). The samples of amniotic fluid, cord blood, neonatal throat swab, and breastmilk samples from six patients tested negative for SARS-CoV-2 (5), suggesting no intrauterine vertical transmission of SARS-CoV-2 in the nine pregnant COVID-19 patients. However, this study enrolled only nine pregnant women with COVID-19, and sample collection was successful in only six infants (5). Another study from Chen et al. reported four pregnant women with COVID-19 (16). All mothers recovered from COVID-19 and had no critical maternal illness, although one mother suffered severe dyspnea after delivery which required respiratory support, and one developed anemia and dyspnea after admission. Of note, none of the three infants whose parents provided consent to be diagnosed tested positive for SARS-CoV-2 from throat swab samples or developed serious clinical symptoms such as fever, cough, or diarrhea. However, two newborns had a rash, which disappeared spontaneously without treatment; a newborn from the mother with placenta previa was considered to suffer from transient tachypnea of the newborn and was supported by non-invasive mechanical ventilation for 3 days. Of note, a study published in *JAMA Pediatrics* indicated three neonates born to a pregnant woman with COVID-19 tested positive for SARS-CoV-2 by qRT-PCR (20). However, as indicated by the medical record, the throat swab sample of the neonate was collected at more than 48 h after delivery. No direct testing of intrauterine tissue samples, such as amniotic fluid, cord blood, or placenta, was collected to detect SARS-CoV-2, which is critical for confirming that the SARS-CoV-2 infection in the neonate was due to intrauterine transmission (20). Therefore, intrauterine SARS-CoV-2 infection remains uncertain.

Recently, two studies published in *JAMA* from separate research teams in China reported that three neonates may have acquired SARS-CoV-2 *in utero* from mothers with COVID-19 based on the elevated IgM antibodies to SARS-CoV-2 in neonates (17, 19).

Specifically, the study from Zeng et al. made a retrospectively review for six pregnant women with COVID-19 (19). All these mothers had mild clinical manifestations and performed cesarean deliveries in their third trimester. Of note, all six newborn babies had a normal 1- and 5-min Apgar score and none of them presented any symptoms of COVID-19. However, serological characteristic results indicated that two infants had SARS-CoV-2-specific IgG and IgM concentrations higher than the normal level (<10 AU/mL). Given that IgM is not usually transferred from mother to fetus because of its larger macromolecular structure under normal conditions (57), the author speculated that the neonates may have been infected with SARS-CoV-2 *in utero* from mothers with COVID-19. However, all neonatal throat swabs and blood samples had negative RT-PCR test results. Moreover, this study is limited by the small sample size, lack of cord blood, placenta, amniotic fluid, mother's vaginal secretions, and breast milk and by incomplete information on the outcome of the infants (19). Similar to the case mentioned above, another study from Lan et al. reported that an infant girl born to a mother with COVID-19 (34 weeks, 2 days of gestation) may have acquired SARS-CoV-2 *in utero* due to the elevated IgM antibodies to SARS-CoV-2 (17). However, both the infant's nasopharyngeal swabs and breast milk sampled 3 days after delivery had a negative RT-PCR test result of SARS-CoV-2. Moreover, all neonates had a normal 1- and 5-min Apgar score. The mother's vaginal secretions obtained at delivery also tested negative for SARS-CoV-2. However, this study is limited by the single case, and the lack of amniotic fluid and placenta. There was also no detailed information regarding the pregnancy stage of the onset of COVID-19.

In summary, there was no positive RT-PCR result in the neonate specimens obtained within 24 h post-birth (5, 14, 16, 17, 19), implying no virologic evidence for congenital infection. However, the serological characteristics of infants reported three neonates with elevated IgM antibodies to SARS-CoV-2 born to a mother with COVID-19, suggesting a possible vertical transmission of SARS-CoV-2 from mother to newborn (17, 19). Indeed, the virologic evidence for supporting the utero transmission should be diagnosed based on RT-PCR test results of the samples from neonates but not IgM detection with a high incidence of its false-positive and false-negative results (58, 59). A reasonable explanation for such inconsistency may be the disruption of the placenta or amniotic barrier caused by the inflammatory mediators from mothers that, induced by SARS-CoV-2, facilitates the cross of IgG and IgM. In detail,

TABLE 2 | Maternal and infant outcome of pregnant women with COVID-19 reported by the indicated study.

Sample size	Pregnancy stage	Delivery manner	Infant outcome	Maternal outcome	RT-PCR results of neonate samples for SARS-CoV-2	References
Nine (only six obtained samples successful)	Third trimester (9/9)	Cesarean section (9/9)	Premature (4/9) Increased myocardial enzymes and creatine kinase-myocardial (1/9)	Lymphopenia (5/9) Fetal distress (2/9)	Positive (0/6) (amniotic fluid, cord blood, throat swab)	(5)
Nine (ten neonates, including two twins)	Third trimester (9/9)	Cesarean section (7/9) Vaginal delivery (2/9)	LGA (1/10) SGA (2/10) Fever (2/10) Thrombocytopenia accompanied by abnormal liver function (2/10) Rapid heart rate (1/10) Vomiting (1/10) Pneumothorax (1/10) Death (1/10)	Fetal distress (9/10)	Positive (0/10) (pharyngeal swab)	(54)
Four	Third trimester (4/4)	Cesarean section (3/4) Vaginal delivery (1/4)	Rashes after birth (2/4) Edema from the mother with cholecystitis (1/4) TTN and required nCPAP after birth from mother with placenta previa (1/4)	Reduced fetal movement (1/4) Anemia and dyspnea (1/4) Lymphopenia (2/4) Increased C-response protein (4/4)	Positive (0/4) (throat swab)	(16)
Six	N/A	Cesarean section at third trimester (6/6)	Increased IgM antibody to SARS-CoV-2 concentration (2/6) Increased IgG antibody to SARS-CoV-2 concentration (5/6) Increased IL-6 concentration (6/6)	Increased IgM/IgG antibody to SARS-CoV-2 concentration (5/6)	Positive (0/6) (throat swabs, blood samples)	(19)
One	Third trimester (1/1)	Cesarean section (1/1)	Increased IgM antibody to SARS-CoV-2 concentration (1/1) Increased IgG antibody to SARS-CoV-2 concentration (1/1)	Increased IgM/IgG antibody to SARS-CoV-2 concentration (1/1)	Positive (0/1) (nasopharyngeal swabs)	(17)
Thirty-three	N/A	Cesarean section (26/33) Vaginal delivery (7/33)	N/A	N/A	Positive (3/33) (nasopharyngeal and anal swabs) All tested positive obtained from Cesarean section	(20)
Three	Third trimester (3/3)	Cesarean section (3/3)	Low birth weight (1/3) Fibrin deposition inside and around the villi with local syncytial nodule increases (3/3)	Increased C-response protein (3/3)	Positive (3/3) (throat swabs)	(55)
Seventeen	Third trimester (17/17)	N/A	Headache (1/17)	N/A	Positive (0/17) (oropharyngeal/ nasopharyngeal combination swab)	(14)
One	Third trimester (1/1)	N/A	Intermittent fever, dry cough, headache, and myalgia (1/1)	A dysplastic and multi-cystic right kidney (1/1)	N/A	(56)

LGA, large-for-gestational-age; SGA, small-for-gestational-age; TTN, transient tachypnea of the newborn; nCPAP, nasal- Continuous Positive Airway Pressure.

the placenta is a barrier to viral infection (60). The damage of the placenta by SARS-CoV-2 may represent an important link in the vertical transmission according to the experience from SARS-CoV. The two placentas from women who were recovering from SARS-CoV infection in the third trimester of pregnancy had abnormal weights and pathologies (53). By contrast, in the

case of COVID-19, whether the placentas from those pregnant while infected with COVID-19 were abnormal or damaged in most of these studies are unknown (5, 16, 17, 19). Indeed, a study reported that there were various degrees of fibrin deposition inside and around the villi with local syncytial nodule increases in three placentas from those pregnant while infected with COVID-19, especially a placenta with massive infarction (55). However, these three placenta samples tested negative for the nucleic acid of SARS-CoV-2, suggesting no virologic evidence in the placenta (55). However, another study revealed that SARS-CoV-2 invasion of the placenta in a woman with COVID-19 in the second trimester through molecular and immunohistochemical assays and electron microscopy (61). Moreover, the public antibody-protein profiles resident in Human Protein Atlas (HPA) revealed enrichment of the SARS-CoV-2 receptor ACE2 in the placenta and ovary (62). Collectively, the possibility of SARS-CoV-2 infection acquired from the uterus cannot be excluded, highlighting the potential for severe morbidity among pregnant women with COVID-19.

An EDITORIAL published in *JAMA* holds that SARS-CoV-2 can theoretically be transmitted in the uterus, especially given that virus' nucleic acid has been detected in blood samples (59). However, nucleic acids do not represent infectious particles. Indeed, it had been revealed that inflammatory mediators, including IL-6, IL-1β, and TNF-α, cause severe dysfunction of the amniotic barrier via decreasing the expression of tight junctions-associated factors claudin-3 and claudin-4 and inducing apoptosis of the amniotic epithelial cells (63). Of note, IL-6 has prominent pro-inflammatory properties (64). IL-6 was significantly increased in all infants from mothers with COVID-19 (19) and the clinical and immunological features suggested that both the concentration of IL-6 and TNF-α are higher in severe COVID-19 patients than in moderate patients (65). The elevated IgM antibody to SARS-CoV-2 in the blood was not observed in all neonates, which may be associated with the different levels of inflammatory mediators among them. Collectively, in addition to the possibility of false-positive and false-negative results of IgM (59), disruption of the placenta barrier and amniotic barrier caused by inflammatory mediators causing the elevated IgM concentration also needs to be further investigated. However, determination of the level of ordinary IgG but not specific to SARS-CoV-2 in neonate blood would be a crucial indicator explaining the disruption of the placenta and amniotic barrier.

POTENTIAL OF MOTHER-TO-CHILD TRANSMISSION OF SARS-CoV-2 BASED ON THE GENERAL ROUTES

In general, the routes of mother-to-child transmission of SARS-CoV-2 mainly include intrauterine vertical transmission, birth, or breastfeeding. There is currently no evidence supporting the intrauterine vertical transmission of both SARS-CoV and SARS-CoV-2 based on the discussion above (4–6, 42, 51). However, all the pregnant women recruited in these studies were in their third trimester. Of note, the effect of SARS-CoV-2 on the infant and maternal outcome may be closely associated with their pregnancy stage during the virus infection, which was observed in both SARS-CoV and rubella (44, 66). Therefore, the possibility of intrauterine transmission in pregnancy with SARS-CoV-2 infection in the first or second trimester of pregnancy cannot be overlooked. The potential damage caused by inflammatory factors (above) also needs to be assessed.

For the transmission during birth, most of the people pregnant while infected with COVID-19 discussed above underwent a cesarean section to deliver the live births in current studies, three neonatal from which exhibited early-onset infection with SARS-CoV-2 (20). By contrast, there were ten patients with COVID-19 who performed vaginal delivery, all infants from which tested negative for SARS-CoV-2 (16, 20, 54). Of note, such low transmitted cases were greatly based on the comprehensive protective methods. Indeed, the samples of vaginal mucosa and shedding in birth canals are crucial samples indicating whether SARS-CoV-2 could be transmitted during vaginal delivery. There were few studies that collected vaginal secretion (1/80) or infant blood (12/80); all tested negative for SARS-CoV-2 (5). Further, as revealed by HPA Tissue Atlas, vaginal secretion expresses virtually no ACE2 (62), implying that SARS-CoV-2 may not infect the tissue. Together, the risk of SARS-CoV-2 transmission by vaginal delivery seems low, although more definitive evidence is required.

Finally, to determine the potential of SARS-CoV-2 transmission via breastfeeding, several studies collected and analyzed breast milk samples (7/63) from patients with COVID-19 pneumonia after their first lactation (5, 17). However, these samples tested negative for SARS-CoV-2, suggesting no evidence supporting the breastfeeding transmission of SARS-CoV-2 (5). Of note, such results were similar to pregnancies with SARS-CoV infection. No viral RNA was detected in the specimens of umbilical cord blood, amniotic fluid, and breast milk from those pregnant while infected with SARS-CoV (48–50). Indeed, the antibody against SARS-CoV can be tested from the umbilical cord and breast milk (48–50). Based on such experiences from SARS-CoV, the antibody against SARS-CoV-2 derived from pregnancy may penetrate the placental barrier to orchestrate antiviral defense in the fetus to combat SARS-CoV-2, which needs to be further determined.

FUTURE PERSPECTIVE: MANAGEMENT GUIDELINES FOR OBSTETRIC PATIENTS AND NEONATES BORN TO MOTHERS WITH SUSPECTED OR PROBABLE COVID-19

In summary, there was a low possibility for mother-to-child transmission of SARS-CoV-2 if adequate protective measures were taken. However, the most crucial point is the potential effect of COVID-19 on maternal and fetal outcomes, rather than whether SARS-CoV-2 can be acquired from the uterus; however, the determination of mother-to-child transmission potential is also important. That said, the effect of COVID-19 on maternal and fetal outcomes should be paid considerable

attention. According to the experience from SARS, although no mother-to-child transmission was observed in SARS, SARS-CoV infection was associated with a high risk of severe maternal illness, maternal death, and spontaneous miscarriages (4, 6, 13, 42–46). Indeed, maternal pneumonia is closely associated with a high incidence of various adverse obstetrical outcomes, including the premature rupture of membranes, preterm labor, intrauterine fetal demise, intrauterine growth restriction, and neonatal death (67–69). Further, although observed in a few cases, COVID-19 may be related to the adverse maternal and infant outcome, including premature births, fetal distress, abnormal fetal liver function, rapid heart rate, etc. (**Table 2**).

To address the safety issues for the obstetrical management and delivery of pregnant women with COVID-19, the advice for those women with SARS-CoV-2 infections during pregnancy and puerperium was prepared by numerous experts from the fields of obstetrics and gynecology, pediatrics, infectious diseases, and critical care (21, 70–75). Similar to the recommendations for the non-pregnant, early isolation, early diagnosis, and early management are still the core criteria of prevention and control transmission for pregnant women with suspected and probable SARS-CoV-2 infection. These recommendations mainly include:

1. At times of COVID-19 outbreaks, all pregnant patients should be assessed for travel history or contact with people from the worst-hit areas of the epidemic within 2 weeks. The definition of a case with suspected COVID-19 should be focused on the clinical symptoms of COVID-19;
2. Pregnant women with labor-confirmed SARS-CoV-2 infection should be treated centrally according to the designation by the department of medical administration. The corresponding risk of adverse pregnancy outcomes contributed by COVID-19 should be informed to the patients;
3. A chest radiograph, especially the computed tomography, is crucial for evaluating the development of COVID-19;
4. Pregnant women with suspected or probable COVID-19 should be informed to the CDC and placed in an isolation room or a negative pressure room if it is available;
5. Prenatal examination and delivery of pregnant women with a SARS-CoV-2 infection should be carried out in negative pressure isolation or on an isolation ward. The management medical staff should wear protective equipment;
6. The timing of childbirth should be based on the specific conditions of the mother and child, the gestational week, and the childbirth conditions. The delivery mode depends on obstetric indication;
7. The specific anesthesia method for SARS-CoV-2-infected pregnant women who require surgical delivery can be general anesthesia and regional anesthesia, which should be performed based on the professional anesthesiologist;
8. Given that the possibility of the intrauterine vertical transmission of SARS-CoV-2 cannot be excluded, all newborns from pregnant patients with suspected or confirmed COVID-19 should be isolated for at least 14 days and should not be breastfed during this period until a SARS-CoV-2 infection is ruled out or cured. The mothers should squeeze milk regularly to ensure lactation. An expert team consisting of obstetricians, nurses, pediatricians, infection control specialists, respiratory therapists, and anesthesiologists should jointly manage pregnant women with COVID-19 and their newborn baby;
9. Pregnant women with COVID-19 should be managed by fixed staff, including obstetrics, neonatal, and other related professionals. The healthcare workers caring for pregnant COVID-19 patients should not care for other patients. All healthcare workers should be daily monitored for fever and cough symptoms of COVID-19. Such individuals should be isolated if they were confirmed or suspected of COVID-19;
10. All health care personnel, trainees, and support staff should be trained in infection control management and containment to prevent the spread of SARS-CoV-2.

AUTHOR CONTRIBUTIONS

YuW, YiW, and JY: conception and design, collection and/or assembly of references, data analysis, interpretation, and manuscript writing. XH and RL: conception and design, manuscript writing, and final approval of manuscript. All authors read and approved the final manuscript.

FUNDING

This work was supported by grants from the Research and Industrialization of Medical Devices for Digital Childbirth (No. 2015B020233010).

REFERENCES

1. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet.* (2020) 395:809–15. doi: 10.1016/S0140-6736(20)30183-5
2. Zhu N, Zhang D, Wang W, Li X, Yang B, Song J, et al. A novel coronavirus from patients with pneumonia in china, 2019. *N Engl J Med.* (2020) 382:727–33. doi: 10.1056/NEJMoa2001017
3. Lu R, Zhao X, Li J, Niu P, Yang B, Wu H, et al. Genomic characterisation and epidemiology of 2019 novel coronavirus: implications for virus origins and receptor binding. *Lancet.* (2020) 395:565–74. doi: 10.1016/S0140-6736(20)30251-8
4. Qiao J. What are the risks of COVID-19 infection in pregnant women? *Lancet.* (2020) 395:760–2. doi: 10.1016/S0140-6736(20)30365-2
5. Chen H, Guo J, Wang C, Luo F, Yu X, Zhang W, et al. Clinical characteristics and intrauterine vertical transmission potential of COVID-19 infection in nine pregnant women: a retrospective review of medical records. *Lancet.* (2020) 395:809–15. doi: 10.1016/S0140-6736(20)30360-3
6. Schwartz D, Graham A. Potential maternal and infant outcomes from (Wuhan) coronavirus 2019-nCoV infecting pregnant women: lessons from SARS, MERS, and other human coronavirus infections. *Viruses.* (2020) 12:194. doi: 10.3390/v12020194

7. Guan WJ, Ni ZY, Hu Y, Liang WH, Ou CQ, He JX, et al. Clinical characteristics of 2019 novel coronavirus infection in China. *N Engl J Med.* (2020) 382:1708–20. doi: 10.1101/2020.02.06.20020974
8. Coronaviridae Study Group of the International Committee on Taxonomy of Viruses. The species Severe acute respiratory syndrome-related coronavirus: classifying 2019-nCoV and naming it SARS-CoV-2. *Nat Microbiol.* (2020) 5:536–44. doi: 10.1038/s41564-020-0695-z
9. Dong E, Du H, Gardner L. An interactive web-based dashboard to track COVID-19 in real time. *Lancet Infect Dis.* (2020) 20:533–4. doi: 10.1016/S1473-3099(20)30120-1
10. Harris J. Influenza occurring in pregnant women: a statistical study of thirteen hundred and fifty cases. *J Am Med Assoc.* (1919) 72:978–80. doi: 10.1001/jama.1919.02610140008002
11. Eickhoff T, Sherman I, Serfling R. Observations on excess mortality associated with epidemic influenza. *Md State Med J.* (1962) 11:115–6.
12. Visscher H, Visscher R. Indirect obstetric deaths in the state of Michigan 1960-1968. *Am J Obstet Gynecol.* (1971) 109:1187–96. doi: 10.1016/0002-9378(71)90664-8
13. Jamieson D, Theiler R, Rasmussen S. Emerging infections and pregnancy. *Emerg Infect Dis.* (2006) 12:1638–43. doi: 10.3201/eid1211.060152
14. Fassett MJ, Lurvey LD, Yasumura L, Nguyen M, Colli JJ, Volodarskiy M, et al. Universal SARS-Cov-2 screening in women admitted for delivery in a large managed care organization. *Am J Perinatol.* (2020). doi: 10.1055/s-0040-1714060
15. Sutton D, Fuchs K, D'Alton M, Goffman D. Universal screening for SARS-CoV-2 in women admitted for delivery. *N Engl J Med.* (2020) 382:2163–4. doi: 10.1056/NEJMc2009316
16. Chen Y, Peng H, Wang L, Zhao Y, Zeng L, Gao H, et al. Infants born to mothers with a new coronavirus (COVID-19). *Front Pediatr.* (2020) 8:104. doi: 10.3389/fped.2020.00104
17. Dong L, Tian J, He S, Zhu C, Wang J, Liu C, et al. Possible vertical transmission of SARS-CoV-2 from an infected mother to her newborn. *JAMA.* (2020) 323:1846–8. doi: 10.1001/jama.2020.4621
18. Li N, Han L, Peng M, Lv Y, Ouyang Y, Liu K, et al. Maternal and neonatal outcomes of pregnant women with COVID-19 pneumonia: a case-control study. *Clin Infect Dis.* (2020). doi: 10.1101/2020.03.10.20033605
19. Zeng H, Xu C, Fan J, Tang Y, Deng Q, Zhang W, et al. Antibodies in infants born to mothers with COVID-19 pneumonia. *JAMA.* (2020) 323:1848–49. doi: 10.1001/jama.2020.4861
20. Zeng L, Xia S, Yuan W, Yan K, Xiao F, Shao J, et al. Neonatal early-onset infection with SARS-CoV-2 in 33 neonates born to mothers with COVID-19 in Wuhan, China. *JAMA Pediatr.* (2020) 174:722–5. doi: 10.1001/jamapediatrics.2020.0878
21. Wang Y, Jin F, Wang R, Li F, Wu Y, Kitazato K, et al. HSP90: a promising broad-spectrum antiviral drug target. *Arch Virol.* (2017) 162:3269–82. doi: 10.1007/s00705-017-3511-1
22. Wu F, Zhao S, Yu B, Chen Y, Wang W, Song ZG, et al. A new coronavirus associated with human respiratory disease in China. *Nature.* (2020) 579:265–9. doi: 10.1038/s41586-020-2008-3
23. Hui D. Epidemic and emerging coronaviruses (severe acute respiratory syndrome and middle east respiratory syndrome). *Clin Chest Med.* (2016) 38:71–86. doi: 10.1016/j.ccm.2016.11.007
24. WHO. *Consensus Document on the Epidemiology of Severe Acute Respiratory Syndrome (SARS).* Geneva: World Health Organization (2003).
25. Zhou P, Yang X, Wang XG, Hu B, Zhang L, Zhang W, et al. A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature.* (2020) 579:270–3. doi: 10.1038/s41586-020-2012-7
26. Wrapp D, Wang N, Corbett KS, Goldsmith JA, Hsieh CL, Abiona O, et al. Cryo-EM structure of the 2019-nCoV spike in the prefusion conformation. *Science.* (2020) 367:1260–3. doi: 10.1126/science.abb2507
27. Xu X, Chen P, Wang J, Feng J, Zhou H, Li X, et al. Evolution of the novel coronavirus from the ongoing Wuhan outbreak and modeling of its spike protein for risk of human transmission. *Sci China Life Sci.* (2020) 63:457–60. doi: 10.1007/s11427-020-1637-5
28. Chan J, Yuan S, Kok KH, To K, Chu H, Yang J, et al. A familial cluster of pneumonia associated with the 2019 novel coronavirus indicating person-to-person transmission: a study of a family cluster. *Lancet.* (2020) 395:514–23. doi: 10.1016/S0140-6736(20)30154-9
29. Phan L, Nguyen T, Luong Q, Nguyen T, Nguyen H, Le H, et al. Importation and human-to-human transmission of a novel coronavirus in Vietnam. *N Engl J Med.* (2020) 382:872–4. doi: 10.1056/NEJMc2001272
30. Rothe C, Schunk M, Sothmann P, Bretzel G, Froeschl G, Wallrauch C, et al. Transmission of 2019-nCoV infection from an asymptomatic contact in germany. *N Engl J Med.* (2020) 382:970–1. doi: 10.1056/NEJMc2001468
31. Wu J, Leung K, Leung G. Nowcasting and forecasting the potential domestic and international spread of the 2019-nCoV outbreak originating in Wuhan, China: a modelling study. *Lancet.* (2020) 395:689–97. doi: 10.1016/S0140-6736(20)30260-9
32. Li Q, Guan X, Wu P, Wang X, Zhou L, Tong Y, et al. Early transmission dynamics in Wuhan, China, of novel coronavirus-infected pneumonia. *N Engl J Med.* (2020) 382:1199–207. doi: 10.1056/NEJMoa2001316
33. Poutanen S, Low D, Henry B, Finkelstein S, Rose D, Green K, et al. Identification of severe acute respiratory syndrome in Canada. *N Engl J Med.* (2003) 348:1995–2005. doi: 10.1056/NEJMoa030634
34. Seto WH, Tsang D, Yung R, Ching TY, Ng T, Ho M, et al. Effectiveness of precautions against droplets and contact in prevention of nosocomial transmission of severe acute respiratory syndrome (SARS). *Lancet.* (2003) 361:1519–20. doi: 10.1016/S0140-6736(03)13168-6
35. Hung L. The SARS Epidemic in Hong Kong—what lessons have we learned? *J R Soc Med.* (2003) 96:374–8. doi: 10.1258/jrsm.96.8.374
36. Sun J, Zhu A, Li H, Zheng K, Zhuang Z, Chen Z, et al. Isolation of infectious SARS-CoV-2 from urine of a COVID-19 patient. *Emerg Microbes Infect.* (2020) 9:991–3. doi: 10.1080/22221751.2020.1760144
37. Leung W, To KF, Chan P, Chan H, Wu A, Lee N, et al. Enteric involvement of severe acute respiratory syndrome - Associated coronavirus infection. *Gastroenterology.* (2003) 125:1011–7. doi: 10.1016/S0016-5085(03)01215-0
38. Minodier L, Charrel R, Ceccaldi PE, Werf S, Blanchon T, Hanslik T, et al. Prevalence of gastrointestinal symptoms in patients with influenza, clinical significance, and pathophysiology of human influenza viruses in faecal samples: what do we know? *Virol J.* (2015) 12:215. doi: 10.1186/s12985-015-0448-4
39. Chen N, Zhou M, Dong X, Qu J, Gong F, Han Y, et al. Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study. *Lancet.* (2020) 395:507–13. doi: 10.1016/S0140-6736(20)30211-7
40. Zumla A, Hui D, Perlman S. Middle east respiratory syndrome. *Lancet.* (2015) 386:995–1007. doi: 10.1016/S0140-6736(15)60454-8
41. WHO. *Clinical Management of Severe Acute Respiratory Infection When Novel Coronavirus (nCoV) Infection is Suspected: Interim Guidance.* Geneva: WHO (2020).
42. Wong S, Chow KM, Swiet M. Severe acute respiratory syndrome and pregnancy. *BJOG Int J Obstet Gynaecol.* (2003) 110:641–2. doi: 10.1046/j.1471-0528.2003.03008.x
43. Ng P, So KW, Leung TF, Cheng F, Lyon D, Wong W, et al. Infection control for SARS in a tertiary neonatal centre. *Arch Dis Childh Fetal Neonatal Ed.* (2003) 88:F405–9. doi: 10.1136/fn.88.5.F405
44. Wong S, Chow K, Leung T, Ng W, Ng T, Shek CC, et al. Pregnancy and perinatal outcomes of women with severe acute respiratory syndrome. *Am J Obstet Gynecol.* (2004) 191:292–7. doi: 10.1016/j.ajog.2003.11.019
45. Ng P, Leung C, Chiu WK, Wong S, Hon EKL. SARS in newborns and children. *Biol Neonate.* (2004) 85:293–8. doi: 10.1159/000078174
46. Zhang JP, Wang YH, Chen LN, Zhang R, Xie YF. Clinical analysis of pregnancy in second and third trimesters complicated severe acute respiratory syndrome. *Zhonghua fu chan ke za zhi.* (2003) 38:516–20.
47. Lam C, Wong S, Leung T, Chow K, Yu W, Wong T, et al. A case-controlled study comparing clinical course and outcomes of pregnant and non-pregnant women with severe acute respiratory syndrome. *BJOG Int J Obstet Gynaecol.* (2004) 111:771–4. doi: 10.1111/j.1471-0528.2004.00199.x
48. Robertson CA, Lowther SA, Birch T, Tan C, Sorhage F, Stockman L, et al. SARS and pregnancy: a case report. *Emerg Infect Dis.* (2004) 10:345–8. doi: 10.3201/eid1002.030736
49. Schneider E, Duncan D, Reiken M, Perry R, Messick J, Sheedy C, et al. SARS in pregnancy. *AWHONN Lifelines.* (2004) 8:122–8. doi: 10.1177/1091592304265557

50. Stockman L, Lowther S, Coy K, Saw J, Parashar U. SARS during pregnancy, United States. *Emerg Infect Dis.* (2004) 10:1689–90. doi: 10.3201/eid1009.040244
51. Yudin M, Steele D, Sgro M, Read S, Kopplin P, Gough K. Severe acute respiratory syndrome in pregnancy. *Obstet Gynecol.* (2005) 105:124–7. doi: 10.1097/01.AOG.0000151598.49129.de
52. Shek CC, Ng PC, Fung GP, Cheng FW, Chan PK, Peiris MJ, et al. Infants born to mothers with severe acute respiratory syndrome. *Pediatrics.* (2003) 112:e254. doi: 10.1542/peds.112.4.e254
53. Ng WF, Wong SF, Lam A, Mak YF, Yao H, Lee KC, et al. The placentas of patients with severe acute respiratory syndrome: a pathophysiological evaluation. *Pathology.* (2006) 38:210–8. doi: 10.1080/00313020600696280
54. Zhu H, Wang L, Fang C, Peng S, Zhang L, Chang G, et al. Clinical analysis of 10 neonates born to mothers with 2019-nCoV pneumonia. *Transl Pediatr.* (2020) 9:51–60. doi: 10.21037/tp.2020.02.06
55. Wang Y, Huang L, Wang Y, Luo W, Li F, Xiao J, et al. Single-cell RNA-sequencing analysis identifies host long noncoding RNA MAMDC2-AS1 as a co-factor for HSV-1 nuclear transport. *Int J Biol Sci.* (2020) 16:1586–603. doi: 10.7150/ijbs.42556
56. Zambrano LI, Fuentes-Barahona IC, Bejarano-Torres DA, Bustillo C, Gonzales G, Vallecillo-Chinchilla G, et al. A pregnant woman with COVID-19 in Central America. *Travel Med Infect Dis.* (2020) 101639. doi: 10.1016/j.tmaid.2020.101639
57. Woo PC, Lau SK, Wong BH, Tsoi HW, Fung AM, Chan KH, et al. Detection of specific antibodies to severe acute respiratory syndrome (SARS) coronavirus nucleocapsid protein for serodiagnosis of SARS coronavirus pneumonia. *J Clin Microbiol.* (2004) 42:2306–9. doi: 10.1128/JCM.42.5.2306-2309.2004
58. Nielsen CM, Hansen K, Andersen HMK, Gerstoft J, Vestergaard BF. An enzyme labelled nuclear antigen immunoassay for detection of cytomegalovirus IgM antibodies in human serum: specific and non-specific reactions. *J Med Virol.* (1987) 22:67–76. doi: 10.1002/jmv.1890220109
59. Kimberlin DW, Stagno S. Can SARS-CoV-2 Infection Be Acquired *In Utero*? *JAMA.* (2020) 323:1788–9. doi: 10.1001/jama.2020.4868
60. Delorme-Axford E, Sadovsky Y, Coyne CB. The placenta as a barrier to viral infections. *Annu Rev Virol.* (2014) 1:133–46. doi: 10.1146/annurev-virology-031413-085524
61. Hosier H, Farhadian SF, Morotti RA, Deshmukh U, Lu-Culligan A, Campbell KH, et al. SARS-CoV-2 infection of the placenta. *J Clin Invest.* (2020) 139569. doi: 10.1172/JCI139569. [Epub ahead of print].
62. Wang Y, Wang Y, Luo W, Huang L, Xiao J, Li F, et al. A comprehensive investigation of the mRNA and protein level of ACE2, the putative receptor of SARS-CoV-2, in human tissues and blood cells. *Int J Med Sci.* (2020) 17:1522–31. doi: 10.7150/ijms.46695
63. Ross MG. Inflammatory mediators weaken the amniotic membrane barrier through disruption of tight junctions. *J Physiol.* (2011) 589:5. doi: 10.1113/jphysiol.2010.202903
64. Moore JB, June CH. Cytokine release syndrome in severe COVID-19. *Science.* (2020) 368:473–74. doi: 10.1126/science.abb8925
65. Chen G, Wu D, Guo W, Cao Y, Huang D, Wang H, et al. Clinical and immunological features of severe and moderate coronavirus disease 2019. *J Clin Invest.* (2020) 130:2620–9. doi: 10.1172/JCI137244
66. Bouthry E, Picone O, Hamdi G, Grangeot-Keros L, Ayoubi JM, Vauloup-Fellous C. Rubella and pregnancy: diagnosis, management and outcomes. *Prenat Diagn.* (2014) 34:1246–53. doi: 10.1002/pd.4467
67. Benedetti T, Valle R, Ledger W. Antepartum pneumonia in pregnancy. *Am J Obstet Gynecol.* (1982) 144:413–7. doi: 10.1016/0002-9378(82)90246-0
68. Berkowitz K, LaSala A. Risk factors associated with the increasing prevalence of pneumonia during pregnancy. *Am J Obstet Gynecol.* (1990) 163:981–5. doi: 10.1016/0002-9378(90)91109-P
69. Madinger N, Greenspoon J, Ellrodt A. Pneumonia during pregnancy: has modern technology improved maternal and fetal outcome? *Am J Obstet Gynecol.* (1989) 161:657–62. doi: 10.1016/0002-9378(89)90373-6
70. Liang H, Acharya G. Novel corona virus disease (COVID-19) in pregnancy: what clinical recommendations to follow? *Acta Obstet Gynecol Scand.* (2020) 99:439–42. doi: 10.1111/aogs.13836
71. Qi H, Luo X, Zheng Y, Zhang H, Li J, Zou L, et al. Safe delivery for COVID-19 infected pregnancies. *BJOG.* (2020) 127:927–9. doi: 10.1111/1471-0528.16231
72. Amatya S, Corr TE, Gandhi CK, Glass KM, Kresch MJ, Mujsce DJ, et al. Management of newborns exposed to mothers with confirmed or suspected COVID-19. *J Perinatol.* (2020) 40:987–96. doi: 10.1038/s41372-020-0695-0
73. Donders F, Lonnee-Hoffmann R, Tsiakalos A, Mendling W, Martinez de Oliveira J, Judlin P, et al. ISIDOG recommendations concerning COVID-19 and pregnancy. *Diagnostics.* (2020) 10:243. doi: 10.3390/diagnostics10040243
74. Almudeer A, Alallah J, AlSaedi S, Anabrees J, Kattan A, AlSalam Z, et al. Recommendations for the management of newborn with suspected or confirmed coronavirus disease-19. *J Clin Neonatol.* (2020) 9:93. doi: 10.4103/jcn.JCN_34_20
75. Bezhenar V, Aylamazyan EK, Zazerskaya IE, Ivanov DO, Arakelyan BV, Bautin A, et al. BRIEF CLINICAL RECOMMENDATIONS: management tactics for pregnant women and women in labor and childbirth with suspected or confirmed COVID-19 infection. (2020). doi: 10.17816/JOWDS20201

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Medicine

REVIEW
published: 07 August 2020
doi: 10.3389/fmed.2020.00444

Plants Metabolites: Possibility of Natural Therapeutics Against the COVID-19 Pandemic

Farhana Rumzum Bhuiyan[1,2]*, *Sabbir Howlader*[3], *Topu Raihan*[4] and *Mahmudul Hasan*[5]

[1] Department of Botany, University of Chittagong, Chittagong, Bangladesh, [2] Laboratory of Biotechnology and Molecular Biology, Department of Botany, University of Chittagong, Chittagong, Bangladesh, [3] Department of Applied Chemistry and Chemical Engineering, University of Chittagong, Chittagong, Bangladesh, [4] Department of Genetic Engineering and Biotechnology, Shahjalal University of Science and Technology, Sylhet, Bangladesh, [5] Department of Pharmaceuticals and Industrial Biotechnology, Sylhet Agricultural University, Sylhet, Bangladesh

COVID-19, a disease induced by SARS-CoV-2 (Severe Acute Respiratory Syndrome Coronavirus-2), has been the cause of a worldwide pandemic. Though extensive research works have been reported in recent days on the development of effective therapeutics against this global health crisis, there is still no approved therapy against SARS-CoV-2. In the present study, plant-synthesized secondary metabolites (PSMs) have been prioritized to make a review focusing on the efficacy of plant-originated therapeutics for the treatment of COVID-19. Plant metabolites are a source of countless medicinal compounds, while the diversity of multidimensional chemical structures has made them superior to treat serious diseases. Some have already been reported as promising alternative medicines and lead compounds for drug repurposing and discovery. The versatility of secondary metabolites may provide novel antibiotics to tackle MDR (Multi-Drug Resistant) microbes too. This review attempted to find out plant metabolites that have the therapeutic potential to treat a wide range of viral pathogens. The study includes the search of remedies belonging to plant families, susceptible viral candidates, antiviral assays, and the mode of therapeutic action; this attempt resulted in the collection of an enormous number of natural therapeutics that might be suggested for the treatment of COVID-19. About 219 plants from 83 families were found to have antiviral activity. Among them, 149 plants from 71 families were screened for the identification of the major plant secondary metabolites (PSMs) that might be effective for this pandemic. Our investigation revealed that the proposed plant metabolites can serve as potential anti- SARS-CoV-2 lead molecules for further optimization and drug development processes to combat COVID-19 and future pandemics caused by viruses. This review will stimulate further analysis by the scientific community and boost antiviral plant-based research followed by novel drug designing.

Keywords: medicinal plants, secondary metabolites, antiviral activities, natural therapeutics/alternative medicine, drug discovery, COVID-19

OPEN ACCESS

Edited by:
Zisis Kozlakidis, International Agency for Research on Cancer (IARC), France

Reviewed by:
Tauqeer Hussain Mallhi, Al Jouf University, Saudi Arabia
Sinosh Skariyachan, St. Pius X College, India

***Correspondence:**
Farhana Rumzum Bhuiyan
farhana.bot@cu.ac.bd

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: *14 April 2020*
Accepted: *06 July 2020*
Published: *07 August 2020*

Citation:
Bhuiyan FR, Howlader S, Raihan T and Hasan M (2020) Plants Metabolites: Possibility of Natural Therapeutics Against the COVID-19 Pandemic. Front. Med. 7:444. doi: 10.3389/fmed.2020.00444

INTRODUCTION

Coronaviruses comprise a group of large, enveloped, positive-sensed, single-stranded RNA viruses that damage the respiratory tract of mammals including humans, bats, and other animals, leading to infections in the respiratory tract (1–5). The Coronavirus disease 2019 (COVID-19), initially called 2019 novel coronavirus (2019-nCoV), is an agile respiratory disease caused by a novel coronavirus primarily detected in Wuhan, China (6, 7). Now, it has spread to 216 countries and caused the death of more than 0.5 million people worldwide and was declared as a pandemic by the World Health Organization (WHO) (8, 9). Seven types of human coronaviruses have been reported so far, including HCoV-OC43, HCoV-229E, HCoV-HKU1, HCoV-NL63, severe acute respiratory syndrome (SARS)-CoV, Middle East respiratory syndrome (MERS-CoV), and 2019-novel coronavirus nCoV (10). Among them, MERS-CoV, SARS-CoV, and nCoV have taken the concern of scientists worldwide. In 2003, the severe acute respiratory syndrome (SARS) outbreak occurred in Guangdong (southern China) (6, 11) which infected 8,000 people and resulted in 800 deaths in 26 countries. Only a decade later, another coronavirus has attacked the world and caused another devastating outbreak, MERS, which infected 2,494 people and caused the deaths of 858 worldwide (12, 13). However, the COVID-19 pandemic caused by SARS CoV-2 resulted in remarkable levels of morbidity and mortality all over the world. Initially China, followed by the USA, Italy, France, Iran, Spain, Russia, Turkey, and the UK became hotspots for SARS CoV-2. The virus hotspot has now moved to Latin America and, at this time, Brazil, Mexico, and Peru are the new hotspots of SARS CoV-2. The important aspects of the pathobiology, a viral response phase, and a hyperbolic host response phase are linked with the morbidity and mortality in COVID-19 patients (14). However, the increased cytokine levels (IL-6, IL-10, and TNF-α), lymphopenia (in CD4+ and CD8+ T cells), and decreased IFN-γ expression in CD4+ T cells are the more risky and possibly life-threatening events related to severe COVID-19 (15–17). The infection rate of COVID-19 is increasing gradually but scientists have not been able to suggest any specific drug, vaccine, or any other certified therapeutic agents against SARS-CoV-2, which consequently leads to the significant morbidity and mortality.

On the other hand, plants have been essential to human welfare for their uses as therapeutics since ancient times (18, 19). According to the WHO, about 80% of the world's population depends on medicinal plants or herbs to fulfill their medicinal needs (20–22). A significant amount of antiviral compounds produced from numerous kinds of plants have been used in many studies (23–25). Researchers all around the world are screening therapeutic drugs from existing antiviral plant secondary metabolites (PSMs) and are also trying to find novel compounds from medicinal plants [(26–159); **Supplementary Table 1**] to avert this global crisis. Plant metabolites can halt the activity of enzymes involved in the replication cycle of CoVs including papain-like protease and 3CL protease, halt the fusion of the S protein of coronaviruses and ACE2 of the host, and also inhibit cellular signaling pathways (123, 144, 160). Screening from existing PSMs, researchers have been trying to find novel compounds from medicinal plants to prevent numerous diseases, including COVID-19 (**Supplementary Table 1**). Therefore, the current manuscript aims to describe potential metabolites from plant sources that have antiviral properties that might be aligned for the alternative approach against COVID-19. Hence, understanding the structure, life cycle, pathogenicity, cell signaling, epidemiology of the recently emerging virus, drug targets, and drug discovery process have become very important issues to find specific/effective therapeutics.

EPIDEMIOLOGY, GENOMIC ORGANIZATION, AND LIFE CYCLE OF SARS CoV-2

In December 2019, SARS CoV-2, one of the most devastating viral outbreaks since SARS CoV and MERS, originated from Wuhan city seafood market in China (161–163). The virus was found to be transmitted through close contact with infected people or through exposure to coughing, sneezing, and respiratory droplets (164, 165). It has already been reported to have spread to 216 countries and caused more than 0.5 million deaths. Brazil is now the new hotspot for SARS CoV-2 after the USA, Russia, France, Italy, Germany, Spain, and the UK, where more than 11 million people are infected (166, 167).

The pleomorphic or spherical shaped SARS COV-2 has a single-stranded RNA genome of 26.4–31.7 kb in length and a crown-like glycoproteins on its surface (168–173). It is more similar to SARS CoV (over 80%) than MERS (174, 175). However, the RNA genome of CoV-2 is considered as one of the largest genomes compared to those of other RNA viruses (176, 177). The largest open reading frame, ORF1ab, encodes non-structural proteins while the remaining ORFs encode four structural proteins, namely the envelope glycoprotein or spike protein (S), envelope (E) protein, membrane (M) protein, and nucleocapsid (N) protein. The S protein mediates attachment to the host cell while the E protein is involved in virus assembly, membrane permeability of the host cell, and virus-host cell interaction. The M protein is known as a central organizer for the coronavirus assembly and the nucleocapsid (N) protein is usually involved in the processing of helical ribonucleocapsid complex, including some accessory proteins (172, 178). Six types of mutations are found in the genome of SARS CoV-2 while three mutations have been reported in *orf 1ab* gene, two mutation in *S* gene, and the final one in the *orf 7b* and *orf 8* (174, 175). Proteomic analysis revealed that SARS CoV-2 is vastly homologous to SARS CoV but two proteins, *orf 8* and *orf 10,* are not homologous to SARS CoV (175). To complete its life cycle, SARS CoV-2 passes into the human body through the nose, mouth, or eyes and then attaches itself to the receptor-binding domain (RBD) using the surface glycoprotein (Spike-protein) of the virion which tries to attach with the hACE2 receptor (179, 180). The entry mechanism of SARS CoV-2 depends on cellular transmembrane serine protease 2 (TMPRSS2) and furin, along with viral receptor ACE2 (180–182). However, after the fusion of the SARS CoV-2 virion particle with the host cell membrane, the envelope and capsid part of the virus are

removed. The virus releases its genetic material (RNA) into the host cell cytoplasm and acts as mRNA for the translation from ORF1a and ORF1ab to produce pp1a and pp1ab polypeptides (169, 183). Subsequently, chymotrypsin-like protease ($3CL^{pro}$) slices these polypeptides into 16 non-structural proteins (NSPs) that are responsible for replication and transcription (184). Then, infected cells produce proteins when they become hijacked by SARS CoV-2. In this situation, the immune system supports the assembly of SARS CoV-2 into new copies of virion particles (185, 186). Freshly synthesized viral nucleic acids and proteins then assemble into the lumen of the ERGIC (Endoplasmic Reticulum Golgi Intermediate Compartment) and leave the cells through exocytosis [(187, 188); **Figure 1**]. Infected cells release virions and infect other human cells.

SARS-CoV-2 viral infection can be divided into three stages: the asymptomatic period, non-severe symptomatic period, and the severe infection stage (17, 189). SARS CoV-2 patients are reported to have a significant amount of cytokines and chemokines; the levels of cytokines are especially highly increased in patients admitted to ICUs (Intensive Care Unit) (190, 191). These significantly high levels are what results in a patient reaching a critical stage. However, the main mediator of SARS CoV-2, the spike glycoprotein, is found in two conformations (192) and the enzyme $3CL^{pro}$ of SARS-CoV-2 share a 99.02% sequence identity with $3CL^{pro}$ of SARS-CoV, which is also highly similar to bat SARS CoV $3CL^{pro}$ (193). SARS CoV-2 binds to the host cell receptor with a higher affinity than SARS CoV (194). SARS CoV-2 has shown some strategic alteration with the substrate-binding site of bat SARS CoV-2 and 12 point-mutations are found in SARS CoV-2 compared to SARS CoV. Mutations disrupt the significant hydrogen bonds and modify the receptor binding site (RBS) of SARS-CoV-2 $3CL^{pro}$. However, the

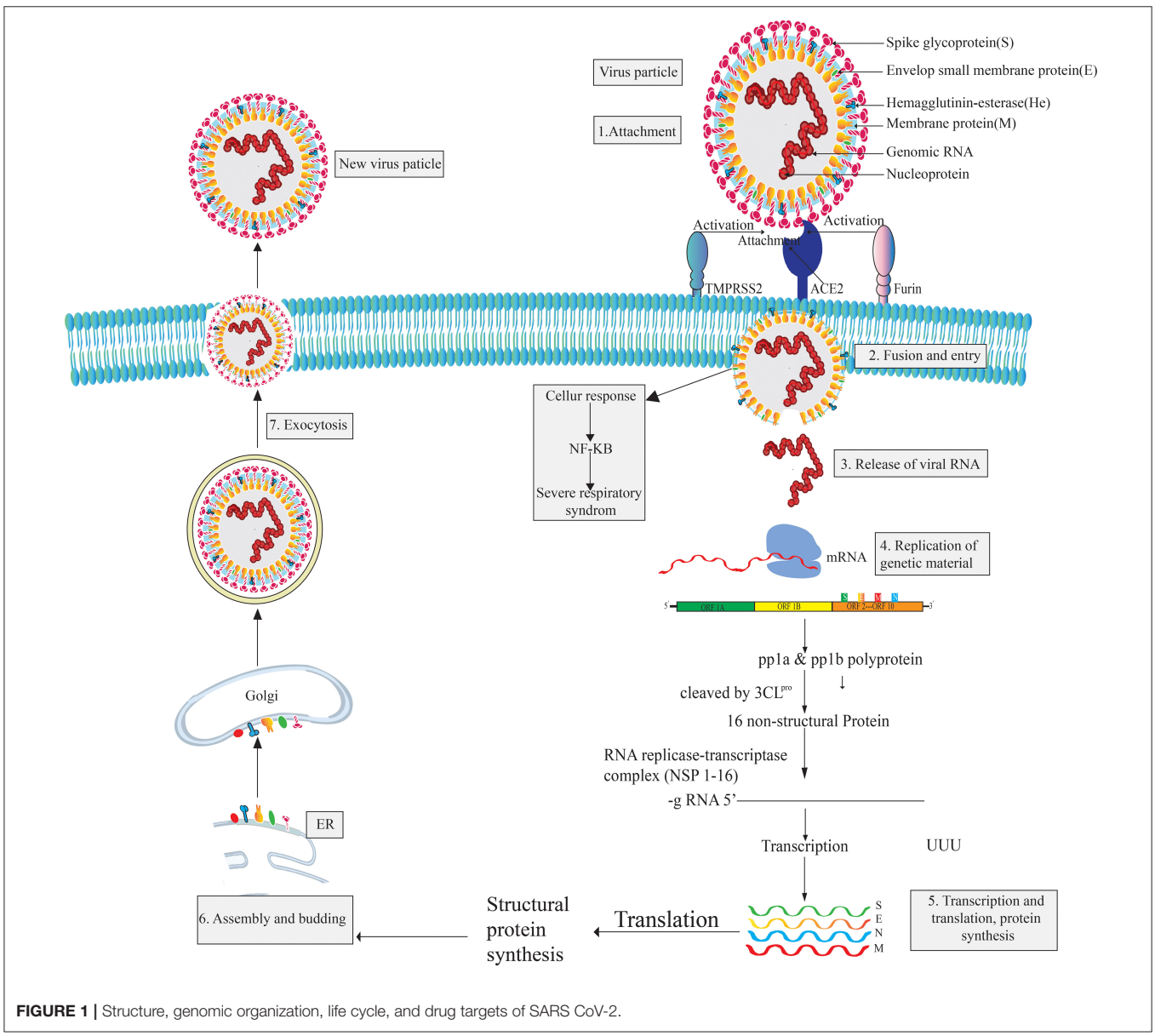


FIGURE 1 | Structure, genomic organization, life cycle, and drug targets of SARS CoV-2.

occurrence of recurrent mutations can lead to new strains with alterations in virulence, which one of the reasons discovering a suitable vaccine to combat SARS CoV-2 is challenging (175, 195).

MAJOR DRUG TARGETS OF SARS CoV-2

A fundamental therapeutic approach to treat multi-viral infections is the interruption of human host-virus interactions (17). The major structural proteins of SARS CoV-2 can be obvious targets for drugs designed against COVID-19. In addition, 16 non-structural proteins (NSPs) can also be considered (169). However, the manifestation of recurrent recombination events is a major hindrance to develop SARS CoV-2 specific vaccines/drugs (176). Up-to-date studies revealed that, though SARS-CoV-2 and SARS-CoV identify a similar receptor (ACE2) in humans (194, 196), there is a noteworthy variation in the antigenicity between SARS-CoV and SARS-CoV-2 which has significance on the development of therapeutic options against this rapidly emerging virus (197). The SARS-CoV-2 spike protein exhibits a higher affinity to the ACE2 receptor in comparison to SARS-CoV, but hACE2 showed a lower binding affinity to RBD (Receptor Binding Domain) of SARS COV-2 when compared to SARS CoV (194, 198). The two most paramount enzymes of SARS CoV-2, proprotein convertase furin- potentiates cell fusion and serine protease TMPRSS2, are responsible for S-protein activation and are propitious drug targets for the treatment of COVID (180, 194, 199).

SARS-CoV-2 AND SEARCHING FOR EFFECTIVE THERAPEUTICS

Though extensive research works are being continued for the development of effective vaccines or drug compounds against SARS-CoV-2, efficacious therapeutics have not yet been attained (200). Moreover, interferon therapies, monoclonal antibodies, oligonucleotide-based therapies, peptides, small-molecule drugs, and vaccines, are regarded as some strategic approaches for controlling or preventing COVID-19 (201, 202). Existing drugs can be used as the first-line treatment for coronavirus outbreaks, but this is not the ultimate solution to eradicate the disease (203). Therefore, the development of therapeutic drugs for the treatment of the COVID-19 outbreak have gathered considerable attention. Scientists from different fields are trying to figure out the way to develop therapeutics. However, experimental implications of drug recombination might be both expensive and time-consuming, whereas computational evaluation may bring about testable hypotheses for systematic drug recombination (174).

PSMs CAN BE EFFECTIVE OVER SYNTHETIC DRUGS AGAINST SARS CoV-2

Though there are approved, repurposed drugs currently in clinical use, there is still an urgent need for specific antiviral therapeutics and vaccines (199). Bioengineered and vectored antibodies and therapies based on cytokines and nucleic acid which target virus gene expression have been found as promising to treat coronavirus infections (204). For example, the repurposing drugs, including favipiravir, remdesivir, lopinavir, ritonavir, nebulized α-interferon, chloroquine, hydroxychloroquine, ribavirin, and interferon (IFN), have been shown to be effective for the treatment of COVID-19. Apart from this, some therapeutics are in clinical trials, such as peptide vaccine (mRNA-1273) (198) and antibody therapies (205). Recently, plasma therapy showed promising results for COVID-19 treatment (206, 207). But, application of these synthetic drugs are not efficient as they exhibit adverse direct or indirect side effects [(208–220); **Table 1**]. In addition, scientists

TABLE 1 | Recently used synthetic drugs and their side effects.

Drug	Side effects	References
Arbidol	Side effects in children include sensitization to the drug	(209)
Darunavir	Liver problems and severe skin reactions or rash	(210)
Flavipir	–	(211)
Hydroxychloroquine	One of the most serious side effects of hydroxychloroquine is a risk of heart rhythm problems, which can result in heart failure and in some cases death. Hydroxychloroquine can upset the stomach. Severe, permanent damage to the retina has been reported with the use of hydroxychloroquine	(212)
Ivermectin	Eye or eyelid irritation, pain, redness, or swelling	(213)
Lopinavir	Drowsiness, dizziness, a bad taste in the mouth, and trouble sleeping	(214)
Loprazolam	Paradoxical increase in aggression, lightheadedness, blood disorders, and jaundice	(215)
Lurasidone	Drowsiness, lightheadedness, weight gain, mask-like facial expression, and agitation	(215)
Oseltamivir	Phlegm-producing cough, wheezing, abdominal or stomach cramps or tenderness, bloating	(216)
Remdesivir	Increased liver enzyme levels that may indicate possible liver damage	(210, 212)
Ribavirin	Allergic reaction, anemia, stabbing chest pain, wheezing	(208)
Ritonavir	Diarrhea, nausea, vomiting, heartburn, stomach pain, dizziness, tiredness	(216)
Salmeterol	Hoarseness, throat irritation, rapid heartbeat, cough, dry mouth/throat, or upset stomach	(218)
Saquinavir	Hyperglycemia, increased bleeding in people with hemophilia, increases in the levels of certain fats	(210)
Talampicillin	–	(215)
Teicoplanin	Maculopapular or erythematous rash and drug-related fever	(219)
Andrographolide (PSM)	–	(220)
Rubitecan	–	(215)

all around the world are trying to find out some prominent drug and multi-epitope vaccine candidates against this deadly virus using various kinds of immuno-informatics approaches (221, 222). Therefore, the urgent need for safe, effective, and inexpensive therapies/drugs with negligible side effects against COVID-19 is imperative.

PSMs are a source of natural antiviral compounds that could be an effective option, as most of them are safer and more cost-effective compared to orthodox drugs (223), though some PSMs are toxic too. The dependency on and popularity of plant-based drugs are increasing day by day (224). Due to the presence of multiple compounds in crude plant extracts, it can be either beneficial or not, depending on the amounts used each time; if properly regulated, better activity might be shown. It was also found that crude extracts can target multiple sites at a time in a virion particle (225). However, this is yet to be tested against SARS-CoV-2. PSMs can affect the disruption of cell membrane functions and structures (226), interference with intermediary metabolisms (227), interruption of DNA/RNA synthesis and function (228), interruption of normal cell communication (quorum sensing) (229), and the induction of coagulation of cytoplasmic constituents (230). Different kinds of plant metabolites act against SARS CoV (**Supplementary Table 1**). Plant-based products affect several key events in the pathogenic process. For example, curcumin is effective for its antineoplastic, anti-proliferative, anti-aging, anti-inflammatory, anti-angiogenic, antiviral and anti-oxidant effects, and can regulate redox status, protein kinases, transcription factors, adhesion molecules, and cytokines in the human body (231). *In silico* analysis revealed that anti-SARS CoV PSMs could be one of the most valuable drug targets against SARS CoV-2 [(232–261); **Table 2**]. A huge amount of plant metabolites have remained unexplored due to the extensive process of isolation of the target compound. Now, various types of modern techniques have been developed for the isolation of lead compounds from crude extracts including maceration, percolation, decoction, reflux extraction, soxhlet extraction, pressurized liquid extraction, supercritical fluid extraction, ultrasound assisted extraction, microwave-assisted extraction, pulsed electric field extraction, enzyme assisted extraction, hydro distillation, and steam distillation (179). These techniques can lead us to find out novel anti-SARS CoV-2 compounds earlier than traditional techniques. In addition, plant metabolomics are used as a tool for the discovery of novel drugs from plant resources (262, 263).

PSMs HAVING ANTIVIRAL PROPERTIES AS ALTERNATIVES TO SYNTHETIC DRUGS AND HOPE FOR CoVID-19

Plants produce diversified low molecular weight PSMs to protect them from different herbivores and microbes (264). Before the discovery of allopathic drugs, these leading natural sources were extensively used for treating several kinds of human diseases (265, 266). Due to the increased resistance of microbial pathogens against allopathic drugs, researchers have now returned to natural resources, focusing especially on plant metabolites, to find out lead compounds to fight against human pathogens (175). Moreover, about 35% of the global medicine market (which accounts for 1.1 trillion US dollars) have been shared by medicinal products prepared using natural plants or herbs (265). Investigations are undergoing for the finding of novel and modern drugs from numerous herbal preparations to fight against this microbial resistance war. Many similarities have been found between SARS CoV and SARS CoV-2 (both of them belong to beta family, containing the same genetic material-RNA, and using the same receptor for viral attachment-ACE2, with an 86% identity and 96% similarity of genome, with almost the same pathogenesis). Thus, previously reported antiviral plant metabolites for SARS CoV can be considered as emerging drug candidates for COVID-19. Right now, the setbacks arising from viral infection around the world have placed budget constraints on researchers trying to discover effective antiviral drugs. However, some PSMs have already shown anti-SARS CoV activity where other antiviral activities are also reported (**Supplementary Table 1**). These results suggest that there is a scope to find alternative medicines and specific compounds. So, plants could be a vital resource in the fight against COVID-19. Our study suggests that around 76 natural metabolites from different plant species can be efficiently active against COVID-19 (**Table 3** and **Supplementary Figure 1**).

PLANT-BASED ANTIVIRAL COMPOUNDS: GROUP BASIS MECHANISM OF ACTION AND PSMs STRUCTURE

A wide variety of antiviral compounds were found from 219 medicinal plants (26–159) belonging to 83 plant families (**Supplementary Table 1**). First and foremost are polyphenols, which contain multiple phenolic rings, and are classified as phenols, flavonoids, lignans, hydroxycinnamic acid, stilbenes, and hydroxybenzoic acid (267). We found polyphenols in numerous plants (**Table 4**) which exerted antiviral activity (269–271) against a wide range of viruses including HIV-1, HIV-2, HSV-1, HSV-2, Influenza virus, Dengue virus, HBV, HCV, Infectious bronchitis virus (IBV), Murbarg virus, Ebola virus, Newcastle disease virus (NDV), Poliomyelitis-1 virus, Lentivirus, and Coronavirus. Polyphenols work against coronaviruses using diverse mechanisms including actuating or inhibiting cellular signaling pathways or halting papain-like protease (PL^{pro}) and 3-chymotripsin-like protease ($3CL^{pro}$) enzyme (269, 272). Some polyphenol compounds (30-(3-methylbut-2-enyl)-30, 4-hydroxyisolonchocarpin, broussochalcone A, 4,7-trihydroxyflavane, broussochalcone B, papyriflavonol A, kazinol A, kazinol B, kazinol F, kazinol J, and broussoflavan A) isolated from *Broussonetia papyrifera* showed promising activity against SARS CoV. Higher efficiency against PL_{pro} as observed by these compounds though activity against M_{pro} or $3CL_{pro}$ is not up to the mark. Specially, papyriflavonol A possesses impressive activity against SARS CoV (IC_{50} 3.7, l M) (272). *In silico* analysis revealed that polyphenols can inhibit SARS CoV-2 Mpro and RdRp effectively (273, 274). In our study, we have

TABLE 2 | Probable drug candidates against SARS CoV-2 obtained through virtual screening.

Drug targets	Major metabolites	References
ANTIVIRAL PSMs THAT CAN INHIBIT SARS CoV-2 AT DIFFERENT TARGET		
Spike protein	Magnoflorine, tinosponone, cirsimaritin, chrysoeriol, vasicinone, quercetin, luteolin	(233)
Spike protein	Epigallocatechingallate (EGCG), curcumin, apigenin, chrysophanol	(234)
Spike protein, main protease	Spike protein, main protease	(235)
Spike protein and ACE-2	Hesperidin, emodin, and chrysin	(236)
Spike protein and ACE-2	Curcumin, nimbin, withaferin A, piperine, mangiferin, thebaine, berberine, and andrographolide	(222)
Spike protein and ACE-2	Chebulagic acid	(237)
Spike protein, MPro, and RdRp	Silybin, withaferin A, cordioside, catechin, and quercetin	(238)
RdRp	Protopine, allocryptopine, and (±) 6-acetonyldihydrochelerythrine	(239)
Main Protease (MPro)	Crocin, digitoxigenin, and b–eudesmol	(240)
Main Protease (MPro)	Oolonghomobisflavan-A, theasinensin D, theaflavin-30-O-gallate	(241)
Main Protease (MPro)	Andrographolide	(220)
Main Protease (MPro)	Hispidin, lepidine E, and folic acid	(242)
Main Protease (MPro)	Ursolic acid, carvacrol, and oleanolic acid	(243)
Main Protease (MPro)	Hypericin, cyanidin 3-glucoside, baicalin, glabridin	(244)
Main Protease (MPro)	Cetylglucopetunidin, isoxanthohumol, and ellagic acid	(245)
Main Protease (MPro)	Benzylidenechromanones	(246)
Main Protease (MPro)	Carnosol, arjunglucoside-I, and rosmanol	(247)
Main Protease (MPro)	Leucoefdin	(248)
Main Protease (MPro)	(1E,6E)-1,2,6,7-tetrahydroxy-1,7-bis(4-hydroxy-3-methoxyphenyl)hepta-1,6-diene-3,5-dione) and (4Z,6E)-1,5-dihydroxy-1,7-bis(4-hydroxyphenyl)hepta-4,6-dien-3-one	(249)
Mpro and ACE2	Quercetin 3-glucuronide-7-glucoside, and Quercetin 3-vicianoside	(250)
Mpro, hACE-2 and RdRp	d-Viniferin, myricitrin, chrysanthemin, myritilin, taiwanhomoflavone A, lactucopicrin 15-oxalate, nympholide A, afzelin, biorobin, hesperidin, and phyllaemblicin B	(251)
Mpro, spike protein, and non-structural proteins (NSP-9, 15)	Arzanol, ferulic acid, genistein, resveratrol, rosmanol	(252)
ACE-2 receptor	Resveratrol, pterostilbene, pinosylvin, piceatannol	(253)
ACE-2 receptor	Isothymol, chloroquine, captopril	(254)
ACE-2 receptor	Resveratrol, quercetin, luteolin, naringenin, zingiberene, and gallic acid	(222)
Envelope protein	Belachinal, macaflavanone E, vibsanol B	(249)
PLpro, 3CLpro	Cryptotanshinone, quercetin, tanshinone IIa, coumaroyltyramine, N-cis-feruloyltyramine	(178)
PLpro, 3CLpro, RdRp, and spike protein	Andrographolide (AGP1), 14-deoxy 11,12-didehydro andrographolide (AGP2), neoandrographolide (AGP3), and 14-deoxy andrographolide (AGP4)	(255)
3CLpro	10-hydroxyusambarensine, cryptoquindoline, 6-oxoisoiguesterin, 22-hydroxyhopan-3-one, cryptospirolepine, isoiguesterin, and 20-epibryonolic acid	(256)
3CLpro	Flavone and coumarine	(210)
3CLpro	Myricitrin, methyl rosmarinat, calceolarioside B, licoleafol, amaranthin, colistin	(191)
6LU7 and 6Y2E proteases	Apigenin, glabridin, glycoumarin, oleanolic acid, glucobrassicin	(257)
Transmembrane protease serine 2 (TMPRSS2)	Withanone and withaferin-A	(258)
Membrane (M) and Envelope (E) proteins	Nimbolin A, nimocin, and cycloartanols	(259)
ANTIVIRAL PSMs THAT CAN INHIBIT SARS CoV-2 AT DIFFERENT LIFE CYCLE		
Viral attachment	Phytoestrogens (diadiazin, genistein, formontein, and biochanin A), chlorogenic acid, linolenic acid, palmitic acid, caffeic acid, caffeic acid phenethyl ester, hydroxytyrosol, cis-p-Coumaric acid, cinnamaldehyde, thymoquinone, and some physiological hormones such as estrogens, progesterone, testosterone, and cholesterol	(260)
Entry	Dihydrotanshinone – 1, desmethoxyreserpine	(241)
Multiplication	Betulinic acid, desmethoxyreserpine, lignan, sugiol	(241)
Viraus–host interaction	Dithymoquinone (DTQ)	(261)

found another widely distributed, low molecular weight phenolic compound named as a flavonoid which showed strong antiviral activity against SARS CoV, Influenza virus, HBV, HSV, HCV, HIV, Dengue virus, Simian virus, Human rotavirus, Bovine viral diarrhea virus, Poliomyelitis-1 virus, Vesicular stomatitis virus (VSV), and Newcastle disease virus (NDV) (**Table 4**). Flavonoid

TABLE 3 | Probable promising secondary metabolites of medicinal plants against COVID-19.

	Compounds	Plant source	Family	References
1.	Diterpneoid	*Andrographis paniculata*	Acanthaceae	(26)
2.	Alkaloids, flavonoids, and coumarins	*Sambucus nigra*	Adoxaceae	(29)
3.	Alkaloids, anthraquinones, glycosides, flavonoids, saponins, phenols, terpenoids, sugar bearing compound, protein, thiols, and inferences	*Iresine herbstii*	Amaranthaceae	(31)
4.	Tannins, Flavonoids, Terpenes, and Saponins	*Anacardium occidentale*	Anacardiaceae	(33)
5.	Tannins, gallic acid, flavonoids like quercetin and quercitrin, phenolics, triterpenes	*Rhus aromatica*	Anacardiaceae	(34)
6.	Gallic acid, quercetin, kaempferol, glycosides	*Rhus parviflora*	Anacardiaceae	(35)
7.	Tannins and flavonoids	*Spondias lutea*	Anacardiaceae	(33)
8.	Flavonoids	*Spondias lutea* L.	Anacardiaceae	(33)
9.	Apigenin and luteolin	*Arisaema tortuosum*	Araceae	(40)
10.	Phenolic acids, flavonoids (apigenin, apigeninglucoside, luteolin, cirsiliol, diosmetin), lignans, terpenic lactones, and alkamides	*Achillea fragrantissima*	Asteraceae	(47, 48)
11.	Flavonoids, clerodane diterpenoids, phenolics, hydroxycinnamic acids	*Baccharis gaudichaudiana DC*	Asteraceae	(49)
12.	Diterpenoids	*Baccharis spicata (Lam.) Baill*	Asteraceae	(49)
13.	Triterpenoids, Steroids	*Bidens subalternans DC*	Asteraceae	(49)
14.	Flavonoid glycosides and caffeoyl quinic acids	*Eupatorium perfoliatum*	Asteraceae	(50)
15.	Flavonoids and terpenes	*Jasonia montana*	Asteraceae	(47)
16.	Phenylpropanoids, flavonoids, essential oils, polyphenols, tannins, triterpenes	*Pluchea sagittalis (Lam.) Cabrera*	Asteraceae	(49)
17.	Silymarin, quercetin, and kaempferol	*Silybum marianum*	Asteraceae	(51)
18.	terpenoids, flavonoids, essential oils	*Tagetes minuta* L.	Asteraceae	(49)
19.	phenolic acids (chlorogenic acids), and sesquiterpene lactones (parthenolide)	*Tanacetum parthenium*	Asteraceae	(52)
20.	Flavonoids, D-glucopyranoside, quercetin, luteolin	*Taraxacum officinale*	Asteraceae	(53)
21.	Flavonoids (apigenin, quercetin, kaempferol, falcarinol, selinene, limonene, and zerumbone)	*Tridax procumbens*	Asteraceae	(55)
22.	Carbohydrates, lipids, proteins, alkaloids, flavonoids, saponins, and organic acids	*Balanites aegyptiaca*	Balanitaceae	(56, 57)
23.	Icariin and quercetin	*Epimedium koreanum Nakai*	Berberidaceae	(58)
24.	Flavonoids (quercetin, isoquercetin, and rutin)	*Capparis sinaica*	Capparaceae	(47, 64)
25.	Tannins, flavonoids, carbohydrates and/or glycosides, resins, sterol, saponins, and alkaloids	*Capparis sinaica*	Capparaceae	(47, 65)
26.	Natural lupane triterpenoids	*Cassine xylocarpa*	Celastraceae	(67)
27.	Pentacyclic lupane-type triterpenoids	*Maytenus cuzcoina*	Celastraceae	(67)
28.	Flavonoids, terpenoids, alkaloids, tannins, glycosides, and saponins	*Combretum adenogonium*	Combretaceae	(72)
29.	Triterpenes, flavonoids, ellagitannins	*Terminalia mollis*	Combretaceae	(56, 73)
30.	Lignans, diterpenes, flavonoids, proanthocyanidins, and sterols	*Taxodium distichum*	Cupressaceae	(75)
31.	Monoterpenoids, sesquiterpenoids, triterpenoids, sterols, alkaloids, flavonoids, and phenolic compounds	*Cyperus rotundus*	Cyperaceae	(76)
32.	Protocatecuic acid, caffeic acid, epicatechin, rutin, resveratrol, quercitin, kaempferol	*Ephedra alata*	Ephedraceae	(47, 77)
33.	Isoflavonoid, indoles, phytosterols, polysaccharides, sesquiterpenes, alkaloids, glucans, and tannins	*Equisetum giganteum*	Equisetaceae	(78)
34.	Triterpenes and steroids	*Euphorbia denticulata*	Euphorbiaceae	(79)
35.	Tannins, diterpenes	*Euphorbia hirta*	Euphorbiaceae	(80)
36.	Diterpenoids, jatrophane-type diterpenoids, and coumarino-type lignoids, lathyrane-type diterpenoids, multifidone, multifidanol, and multifidenol	*Jatropha multifida*	Euphorbiaceae	(82)
37.	Flavonoid and polyphenol	*Acacia arabica*	Fabaceae	(83)
38.	Luteolin and vitexin	*Aspalathus linearis*	Fabaceae	(85)
39.	Saponins and flavonoids	*Vachellia nilotica*	Fabaceae	(87)
40.	Catechin, kaempferol, quercetin, 3,4′,7-trihydroxyl-3′,5-dimethoxyflavone, rutin, isorhamnetin, epicatechin, afzelechin, epiafzelechin, mesquitol, ophioglonin, aromadendrin, and phenol	*Acacia catechu*	Fabaceae	(88)
41.	Flavonoids, phenolics, and tannins	*Quercus persica*	Fagaceae	(90)
42.	Phenolic, flavonoid, and flavonol compounds	*Quercus persica*	Fagaceae	(90)
43.	Gallic acid, protocatechuic acid, corilagin, geraniin, ellagic acid, kaempferitrin, kaempferol 7-O-rhamnoside, quercetin, kaempferol	*Geranium thunbergii*	Geraniaceae	(91)
44.	Flavonoids (orientin and vicenin)	*Ocimum sanctum*	Lamiaceae	(26, 99)
45.	Terpenoid and polyphenol	*Ocimum sanctum*	Lamiaceae	(83)

(Continued)

TABLE 3 | Continued

	Compounds	Plant source	Family	References
46.	Baicalin, flavonoids	*Scutellaria baicalensis*	Lamiaceae	(104)
47.	Opuntin B, triterpene saponin, seroids, and phenylethanoids	*Lindernia crustacea*	Linderniaceae	(107)
48.	Quercetin 3-O-methyl ether (3MQ) and strychnobiflavone (SBF)	*Strychnos pseudoquina*	Loganiaceae	(108)
49.	Alkaloids, flavonoids, tannins, volatile oils, and glycosides	*Cissampelos pareira Linn*	Menispermaceae	(113)
50.	Flavonoids, tannins, terpenes, saponins, and nitrogenous compounds	*Artocarpus integrifolia*	Moraceae	(33)
51.	Flavonoids, rutin, kaempferol 3-O-rutinoside, and kaempferol 3-O-robinobioside	*Ficus benjamina*	Moraceae	(114)
52.	N-arginine, luteolin, caffeic acid	*Ficus carica*	Moraceae	(115)
53.	Flavonoids, tannins, saponins, alkaloids, and steroids/triterpenoids	*Ficus religiosa*	Moraceae	(116)
54.	Tannins, flavonoid, saponin, glycoside	*Ficus sycomorus*	Moraceae	(56, 118)
55.	Alkaloids, tannins, phenolics, and saponins	*Moringa peregrina*	Moringaceae	(47)
56.	Flavonoids	*Myristica fragrans*	Myristicaceae	(33)
57.	Tannins and flavonoids	*Psidium guajava*	Myrtaceae	(33)
58.	Sesquiterpenes, monoterpenes, hydrocarbon, and phenolic compounds, eugenyl acetate, eugenol, and β-caryophyllene	*Syzygium aromaticum* L.	Myrtaceae	(119)
59.	Paeoniflorin, monoterpene glycosides, albiflorin, benzoylpaeoniflorin, gallic acid, ethyl gallate	*Paeonia delavayi*	Paeoniaceae	(121)
60.	Flavonoids, tomentin A, B, C, D, and E	*Paulownia tomentosa*	Paulowniaceae	(123)
61.	Highly oxygenated norbisabolane sesquiterpenoids, phyllanthacidoid acid, methyl ester	*Phyllanthus acidus*	Phyllanthaceae	(124)
62.	Alkaloids, flavonoids, lignans, phenols, and terpenes	*Phyllanthus amarus*	Phyllanthaceae	(125)
63.	Geraniin, rutin, gallic acid, caffeolquinic acid, corilagen, galloylglucopyronoside, digalloylglucopyronoside, and quercetin glucoside	*Phyllanthus amarus*	Phyllanthaceae	(126)
64.	Geraniin, rutin, gallic acid, caffeolquinic acid, corilagen, galloylglucopyronoside, digalloylglucopyronoside, and quercetin glucoside	*Phyllanthus niruri*	Phyllanthaceae	(126)
65.	Trigalloylglucopyronoside, quercetin rhamnoside, geraniin, rutin, gallic acid, caffeolquinic acid, corilagen, galloylglucopyronoside, digalloylglucopyronoside, and quercetin glucoside	*Phyllanthus urinaria*	Phyllanthaceae	(126)
66.	Quercetin rhamnoside, geraniin, rutin, gallic acid, caffeolquinic acid, corilagen, galloylglucopyronoside, digalloylglucopyronoside, and quercetin glucoside	*Phyllanthus watsonii*	Phyllanthaceae	(126)
67.	Plumbagin, allicin, carbohydrates, flavonoids, proteins, saponins, fats and oils, alkaloids, steroids, phenols, and tannins	*Plumbago indica*	Plumbaginaceae	(129)
68.	Flavonoids (catechin, hyperoside, quercitrin, quercetin, and rutin), tannins, and triterpenoids	*Agrimonia pilosa*	Rosaceae	(135)
69.	Hydroxycinnamic acids, eriodictyol, isorhamnetin, quercetin, kaempferol, isorhamnetin, epicatechin, catechin	*Prunus dulcis*	Rosaceae	(136)
70.	Saponins, flavonoids, and alkaloids	*Pavetta tomentosa*	Rubiaceae	(138)
71.	Saponins, flavonoids, and alkaloids	*Tarenna asiatica*	Rubiaceae	(138)
72.	Triterpenes, tannins, flavonoids, and carbohydrates	*Dimocarpus longan*	Sapindaceae	(140)
73.	Organic acids, terpenoids, and flavonoids	*Illicium verum Hook. f.*	Schisandraceae	(142)
74.	Nilocitin, ellagic acid, gallic acid, flavonoids	*Tamarix nilotica*	Tamaricaceae	(47, 143)
75.	Diterpenoids, biflavonoids (biflavone amentoflavone, apigenin, luteolin, and quercetin)	*Torreya nucifera*	Taxaceae	(144)
76.	Friedelolactones, 2β-hydroxy-3, 4-seco-friedelolactone-27-oic acid flavonoids, coumarins, terpenoids, sterols, polypeptides	*Viola diffusa*	Violaceae	(147)

type compounds, such as apigenin and quercetin, showed activity against SARS CoV virion particles through the inhibition of M_{pro} enzymes with an IC_{50} of 38.4 ± 2.4 μM and 23.8 μM, respectively (144, 150, 275). According to *in silico* analysis, flavonoid compounds can terminate the activity of M_{pro} of SARS CoV-2 (276, 277).

Alkaloids are another class of natural organic compounds which are classified into several groups based on their heterocyclic ring, such as tropanes, pyrrolidines, isoquinoline purines, imidazoles, quinolizidines, indoles, piperidines, and pyrrolizidines (278). Alkaloids are very promising against HIV-1, HSV-1, HSV-2, DNV, VSV, Influenza virus, and Newcastle disease virus (NDV) (**Table 4**). Different kinds of alkaloids showed anti-SARS activity including emetine, Ipecac, Macetaxime, tylophorine, and 7-methoxy cryptopleurine, through the inhibition of protease enzyme, RNA synthesis, and protein synthesis (244, 279). In addition, some alkaloids act against SARS CoV as a nucleic acid intercalating agent such as tetrandrine, fangchinoline, cepharanthine, and lycorine through degrading nucleic acids and inhibiting spike and nucleocapsid proteins (280). Virtual screening analysis revealed that 10-Hydroxyusambarensine and Cryptoquindoline—two alkaloid compound isolated from African medicinal plants showed anti-SARS CoV and anti-SARS CoV-2 activity through inhibition of their M_{pro} (256). Chloroquine, a derivative of alkaloid, is found to be active against anti-SARS CoV-2 (281). So,

TABLE 4 | Major group basis antiviral PSMs obtained from medicinal plants.

Major compounds	Plant source	Family	Target pathogen	References
Polyphenols	*Avicennia marina*	Acanthaceae	Human immunodeficiency virus (HIV) and herpes simplex virus (HSV)	(27)
	Sambucus nigra	Adoxaceae	Dengue virus serotype-2 (DENV-2)	(29)
	Sambucus nigra	Adoxaceae	Infectious bronchitis virus (IBV)—chicken coronavirus	(30)
	Iresine Herbstii	Amaranthaceae	Newcastle disease virus (NDV)	(31)
	Anacardium occidentale	Anacardiaceae	Simian (SA-11) virus	(33)
	Artocarpus integrifolia	Moraceae	(SA-11) and human (HCR3) rotaviruses	(33)
	Myristica fragrans	Myristicaceae	Human (HCR3) rotaviruses	(33)
	Psidium guajava	Myrtaceae	Simian (SA-11) virus	(33)
	Spondias lutea	Anacardiaceae	Human (HCR3) rotaviruses	(33)
	Spondias lutea L.	Anacardiaceae	Simian (SA-11) and human (HCR3) rotaviruses	(33)
	Rhus aromatica	Anacardiaceae	HSV-1 and HSV-2	(34)
	Rhus aromatica	Anacardiaceae	HSV-1 and HSV-2	(34)
	Rhus parviflora	Anacardiaceae	HIV-1	(35)
	Schinus terebinthifolia	Anacardiaceae	HSV-1	(36)
	Arisaema Tortuosum	Araceae	Acyclovir-resistant HSV-2 and HSV-1	(40)
	Jasonia montana	Asteraceae	Poliomyelitis-1 virus	(47)
	Baccharis gaudichaudiana DC	Asteraceae	Bovine viral diarrhea virus, HSV-1, Poliovirus type 2 (PV-2), and vesicular stomatitis virus (VSV)	(49)
	Pluchea sagittalis (Lam.) Cabrera	Asteraceae	Bovine viral diarrhea virus (BVDV) (HSV-1), poliovirus type 2 (PV-2), and vesicular stomatitis virus (VSV)	(49)
	Tagetes minuta L	Asteraceae	Bovine viral diarrhea virus, HSV-1, poliovirus type 2 (PV-2), and vesicular stomatitis virus	(49)
	Eupatorium perfoliatum	Asteraceae	Influenza A virus (IAV) H1N1	(50)
	Silybum marianum	Asteraceae	Chikungunya virus (CHIKV), hepatitis C virus (HCV)	(51)
	Tanacetum parthenium	Asteraceae	HSV-1	(52)
	Taraxacum officinale	Asteraceae	HCV	(53)
	Senna angustifolia	Fabaceae	Dengue virus serotype-2 (DENV-2)	(55)
	Tridax procumbers	Asteraceae	Dengue virus serotype-2 (DENV-2)	(55)
	Vernonia cinerea	Asteraceae	Dengue virus serotype-2 (DENV-2)	(55)
	Epimedium koreanum Nakai	Berberidaceae	Porcine epidermic diarrhea virus (PEDV)	(58)
	Canarium album (Lour.)	Burseraceae	Influenza A virus (IAV)	(62)
Polyphenols	*Cistus incanus*	Cistaceae	HIV (clinical HIV-1 and HIV-2) and Filoviruses, Ebola, and Marburg virus	(69)
	Combretum adenogonium	Combretaceae	HIV-1	(72)
	Cornus canadensis	Cornaceae	HSV-1	(74)
	Taxodium distichum	Cupressaceae	Influenza A and B viruses	(75)
	Cyperus rotundus	Cyperaceae	HSV-1, HBV	(76)
	Equisetum giganteum	Equisetaceae	HSV-2	(78)
	Euphorbia hirta	Euphorbiaceae	HIV-1, HIV-2, SIV mac 251	(80)
	Euphorbia sikkimensis	Euphorbiaceae	HIV-1	(81)
	Acacia arabica	Fabaceae	Influenza A virus H9N2	(83)
	Aspalathus linearis	Fabaceae	Rhesus rotavirus (RRV), simian rotavirus (SA-11) infection	(85)
	Vachellia nilotica	Fabaceae	HSV-2	(87)
	Acacia catechu	Fabaceae	HIV-1	(88)
	Acacia catechu	Fabaceae	HIV-1	(88)
	Quercus persica	Fagaceae	HSV-I	(90)
	Geranium thunbergii	Geraniaceae	Influenza virus, H1N1, H3N2, influenza type B	(91)
	Pelargonium sidoides	Geraniaceae	HIV-1	(92)
	Ribes nigrum	Grossulariaceae	Influenza A virus	(94)
	Hamamelis virginiana	Hamamelidaceae	Influenza A virus and human papillomavirus	(95)
	Prunella vulgaris	Lamiaceae	Lentivirus	(101)
	Scutellaria baicalensis	Lamiaceae	RSV, HIV, Influenza, and Dengue viruses	(104)

(Continued)

TABLE 4 | Continued

Major compounds	Plant source	Family	Target pathogen	References
	Strychnos pseudoquina	Loganiaceae	HSV-1 (KOS strain) and HSV-2 (333 strain)	(108)
	Punica granatum	Lythraceae	HSV-2	(109)
	Magnolia officinalis	Magnoliaceae	Dengue virus type 2	(111)
	Cissampelos pareira Linn	Menispermaceae	Dengue virus types 1-4 (DENV-1-4)	(113)
	Ficus benjamina	Moraceae	HSV-1 and HSV-2), varicella zoster virus (VZV	(114)
	Ficus carica	Moraceae	HSV-1, HSV-1, ECV-11, and ADV, influenza virus	(115)
	Ficus religiosa	Moraceae	HSV-2	(116)
	Syzygium aromaticum L.	Myrtaceae	HSV and HCV	(119)
	Paulownia tomentosa	Paulowniaceae	SARS-CoV papain-like protease (PLpro)	(123)
	Phyllanthus amarus	Phyllanthaceae	Acyclovir-resistant HSV strains, hepatitis B virus (HBV), HCV, and HIV	(126)
Polyphenols	*Phyllanthus niruri*	Phyllanthaceae	Acyclovir-resistant HSV strains, hepatitis B virus (HBV), HCV, HIV	(126)
	Phyllanthus urinaria	Phyllanthaceae	Acyclovir-resistant HSV strains, hepatitis B virus (HBV), HCV and HIV	(126)
	Phyllanthus watsonii	Phyllanthaceae	Acyclovir-resistant HSV strains, hepatitis B virus (HBV), HCV, and HIV	(126)
	Limonium sinense	Plumbaginaceae	HCV	(128)
	Plumbago indica	Plumbaginaceae	Influenza A (H1N1)	(129)
	Agrimonia pilosa	Rosaceae	Influenza viruses (H1N1 and H3N2)	(135)
	Prunus dulcis	Rosaceae	HSV-1	(136)
	Pavetta tomentosa	Rubiaceae	Dengue virus (DENV)	(138)
	Aegle marmelos	Rutaceae	Human coxsackieviruses B1-B6, rotavirus SA-11	(139)
	Dimocarpus longan	Sapindaceae	HCV (genotype 2a strain JFH1)	(140)
	Torreya nucifera	Taxaceae	SARS-CoV 3CLpro	(144)
	Viola diffusa	Violaceae	Hepatitis B virus	(147)
	Alpinia katsumadai	Zingiberaceae	influenza virus type A	(148)
	Illicium verum Hook. f.	Schisandraceae	Grouper iridovirus infection (GIV)	(190)
	Camellia sinensis	Theaceae	HIV, HTLV-1, HCV, influenza, and HBV	(145, 146)
	Ocimum sanctum	Lamiaceae	Dengue virus serotype-1 (DENV-1)	(26, 99)
	Achillea fragrantissima	Asteraceae	Poliomyelitis-1 virus	(47, 48)
	Ephedra alata	Ephedraceae	HSV	(47, 77)
	Tamarix nilotica	Tamaricaceae	HSV	(47, 143)
	Moringa peregrina	Moringaceae	HSV	(47, 189)
	Capparis sinaica	Capparaceae	Avian influenza strain H5N1	(47, 64)
	Ficus sycomorus	Moraceae	HSV-1	(56, 118)
	Balanites aegyptiaca	Balanitaceae	VSV	(56, 57)
	Terminalia mollis	Combretaceae	HSV-0	(56, 73)
	Tuberaria lignosa	Cistaceae	HIV	(70, 71)
	Anthemis hyaline	Asreraceae	SARS-CoV	(152)
	Alnus japonica	Betulaceae	SARS-CoV	(59)
	Cassia tora	Fabaceae	SARS-CoV	(156)
	Psoralea corylifolia	Fabaceae	SARS-CoV	(150)
	Taxillus chinensis	Loranthaceae	SARS-CoV	(268)
Polyphenols	*Citrus sinensis*	Rutaceae	SARS-CoV	(152)
	Polygonum multiflorum	Polygonaceae	SARS-CoV	(158)
	Rheum officinale	Polygonaceae	SARS-CoV	(158)
	Rheum palmatum	Polygonaceae	SARS-CoV	(159)
	Citrus sinensis	Rutaceae	SARS-CoV	(152)
Alkaloids	*Sambucus nigra*	Adoxaceae	Dengue virus serotype-2 (DENV-2)	(29)
	Iresine Herbstii	Amaranthaceae	Newcastle disease virus (NDV)	(31)
	Combretum adenogonium	Combretaceae	HIV-1	(72)
	Cyperus rotundus	Cyperaceae	HSV-1, HBV	(76)

(Continued)

TABLE 4 | Continued

Major compounds	Plant source	Family	Target pathogen	References
	Equisetum giganteum	Equisetaceae	HSV-2	(78)
	Cissampelos pareira Linn	Menispermaceae	Dengue virus types 1-4 (DENV-1-4)	(113)
	Ficus religiosa	Moraceae	HSV-2	(116)
	Phyllanthus amarus	Phyllanthaceae	HCV	(125)
	Plumbago indica	Plumbaginaceae	Influenza A (H1N1)	(129)
	Pavetta tomentosa	Rubiaceae	Dengue virus (DENV)	(138)
	Tarenna asiatica	Rubiaceae	Dengue virus (DENV)	(138)
	Moringa peregrina	Moringaceae	HSV	(47, 189)
	Capparis sinaica	Capparaceae	HSV	(47, 65)
	Balanites aegyptiaca	Balanitaceae	VSV	(56, 57)
	Lycoris radiata	Amaryllis	SARS-CoV	(151)
	Acanthopanacis cortex	Araliaceae	SARS-CoV	(134)
Saponins	*Iresine Herbstii*	Amaranthaceae	Newcastle disease virus (NDV)	(31)
	Anacardium occidentale	Anacardiaceae	Simian (SA-11) virus	(33)
	Panax ginseng	Araliaceae	RSV	(41)
	Panax ginseng	Araliaceae	Murine norovirus (MNV) and feline calicivirus (FCV)	(42)
	Balanites aegyptiaca	Balanitaceae	VSV	(56, 57)
	Capparis sinaica	Capparaceae	HSV	(47, 65)
	Combretum adenogonium	Combretaceae	HIV-1	(72)
	Vachellia nilotica	Fabaceae	HSV-2	(87)
	Lindernia crustacea	Linderniaceae	Epstein–Barr virus (EBV)	(107)
	Artocarpus integrifolia	Moraceae	(SA-11) and human (HCR3) rotaviruses	(33)
	Ficus religiosa	Moraceae	HSV-2	(116)
Saponins	*Ficus sycomorus*	Moraceae	HSV-1	(56, 118)
	Moringa peregrina	Moringaceae	HSV	(47, 189)
	Plumbago indica	Plumbaginaceae	Influenza A (H1N1)	(129)
	Pavetta tomentosa	Rubiaceae	Dengue virus (DENV)	(138)
	Tarenna asiatica	Rubiaceae	Dengue virus (DENV)	(138)
Terpenoids	*Andrographis paniculata*	Acanthaceae	Dengue virus serotype-1 (DENV-1)	(26)
	Baccharis gaudichaudiana DC	Asteraceae	Bovine viral diarrhea virus, HSV-1, Poliovirus type 2 (PV-2), and vesicular stomatitis virus (VSV)	(49)
	Baccharis spicata (Lam.) Baill	Asteraceae	Bovine viral diarrhea virus (BVD), HSV-1, poliovirus type 2 (PV-2), and vesicular stomatitis virus (VSV)	(49)
	Taxodium distichum	Cupressaceae	Influenza A and B viruses	(75)
	Euphorbia hirta	Euphorbiaceae	HIV-1, HIV-2, SIV mac 251	(80)
	Jatropha multifida	Euphorbiaceae	Influenza A H1N1 virus	(82)
	Torreya nucifera	Taxaceae	SARS-CoV 3CLpro	(144)
	Agrimonia pilosa	Rosaceae	Influenza viruses (H1N1 and H3N2)	(135)
	Tripterygium regelii	Celastraceae	SARS-CoV	(144)
	Gentiana scabra	Gentianaceae	SARS-CoV	(156)
Carbohydrates	*Panax ginseng*	*Araliaceae*	Human rotavirus	(33)
	Panax notoginseng	*Araliaceae*	Influenza A H1N1 virus	(43)
	Equisetum giganteum	*Equisetaceae*	HSV-2	(78)
	Prunella vulgaris	*Lamiaceae*	HSV-1 and HSV-2 antigens virus antigen in Vero cells	(100)
	Prunellae Spica	*Lamiaceae*	Herpes simplex virus (HSV)	(102)
	Laminaria japonica	*Laminariaceae*	RSV	(105)
	Plumbago indica	*Plumbaginaceae*	Influenza A (H1N1)	(129)
	Ardisia chinensis Benth	*Primulaceae*	Coxsackie B3 Virus	(131)
	Capparis sinaica	*Capparaceae*	HSV	(47, 65)
	Balanites aegyptiaca	*Balanitaceae*	VSV	(56, 57)
	Carissa edulis	*Apocynaceae*	herpes simplex virus, chickenpox, and shingles	(38)

some PSMs as alkaloids can be alternative drug targets for COVID-19 (280).

Another class of PSMs, saponins (amphipathic glycosides), are found ubiquitously in plants which showed antiviral activities against Newcastle disease virus (NDV), Simian (SA-11) virus, Murine norovirus (MNV) and Feline calicivirus (FCV), RSV, VSV, HSV-1,HSv-2, HIV-1, Epstein–Barr virus (EBV), (SA-11) and human (HCR3) rotaviruses, Influenza virus, and Dengue virus (**Table 4**). Plants produce five carbon isoprene derived terpenes which are the largest and most diverse group of PSM. They are classified by monoterpenes, diterpenes, triterpenes, sesterterpenes, hemi terpenes, and sesquiterpenes (282). They exhibited antiviral activity against Bovine viral diarrhea virus, HSV-1, Poliovirus type 2 (PV-2) and vesicular stomatitis virus (VSV), Dengue virus serotype-1 (DENV-1), Influenza A and B viruses, HIV-1, HIV-2, SIV mac 251, and SARS-CoV (**Table 4**). Ten diterpenes, two sesquiterpenes, and two triterpenes showed anti-SARS activity with IC_{50} of 3–10 μM (283). *In silico* analysis also revealed that terpene Ginkgolide A can strongly inhibit SARS CoV-2 protease enzyme (284). Carbohydrates, mainly classified as monosaccharides, disaccharides, polysaccharides, and oligosaccharides (282), are found as antiviral agent against Human rotavirus, Influenza A virus, HSV-1, HSV-2, Herpes simplex virus (HSV), RSV, Coxsackie B3 Virus, and VSV [(285); **Table 4**]. Acyclovir is an FDA (Food and Drug Administration) approved antiviral drug which is obtained from *Carissa edulis* (**Supplementary Table 1**). It is mainly used for herpes simplex virus, chickenpox, and shingles. The group basis structure of some major compounds can be found in **Table 5**.

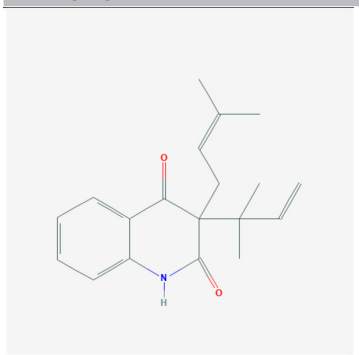
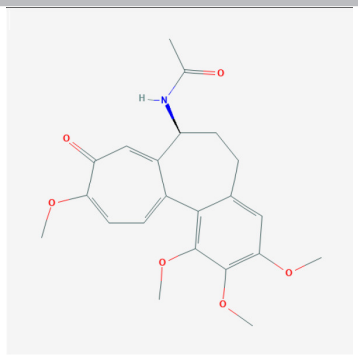
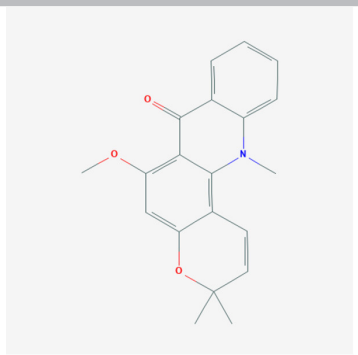
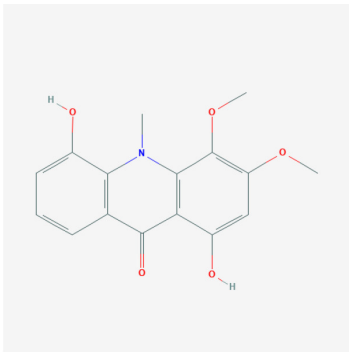
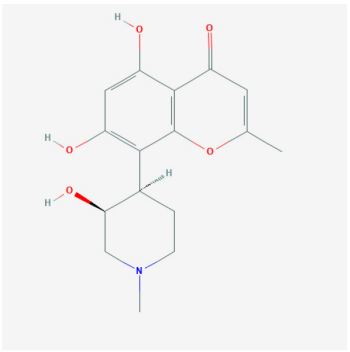
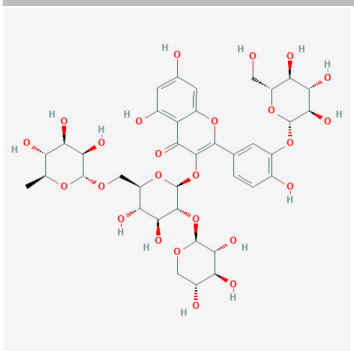
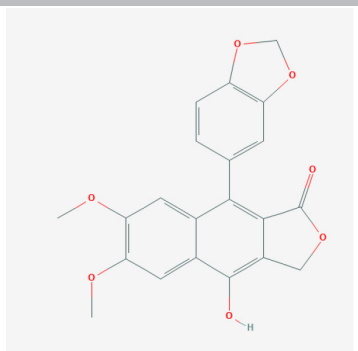
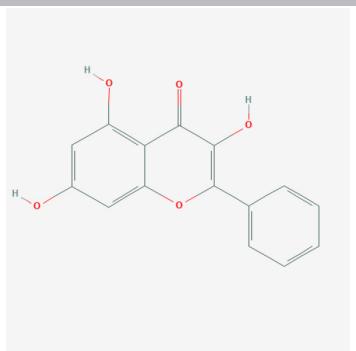
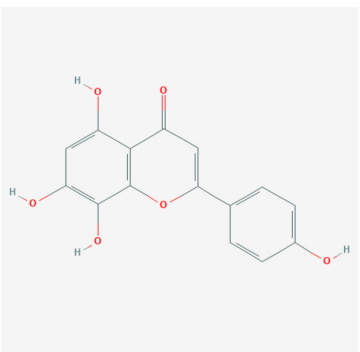
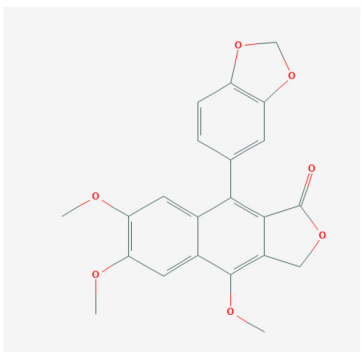
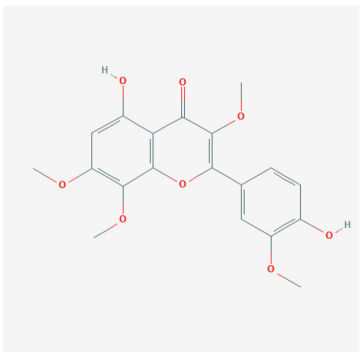
DRUG DISCOVERY FROM PSMs: ADDRESSING THE MAJOR CHALLENGES TOWARD FUTURE INSIGHTS

Drug discovery from plant metabolites refers to the extraction and purification of active ingredients from conventional cures. Natural plant products comprise complicated chemical structures which differ according to their numerous species. There are several classes of PSMs which are responsible for the biological activities of herbal medicines. PSMs exert their actions on molecular targets that differ from one case to the other. These targets may be enzymes, mediators, transcription factors, or even nucleic acids (286). Good knowledge of the chemical composition of plants leads to a better understanding of their possible and specific medicinal value. Drug discovery and development have become a wide interdisciplinary field over recent decades and many factors are involved in the successful evolution from a bioactive compound into a potential drug [(287, 288); **Figure 2**]. When existing methods with advanced technologies are applied, it can lead to a modern revelation of drugs, benefitting medicinal purposes (223, 289). The development of modern technologies has streamlined the screening of natural products in discovering new drugs. Research for drug discovery must create robust and prudent lead molecules, which is progressed from a screening hit to a drug candidate through structural elucidation and structure recognizable proof available from high throughput technology like GC–MS, NMR, IR, HPLC, and HPTLC. Utilizing these advanced technologies gives us an opportunity to perform research in screening novel molecules employing a computer program and database to set up common items as a major source for drug discovery. It finally leads to lead structure discovery. Powerful new technologies are revolutionizing natural herbal drug discovery (223). Steps associated with the drug discovery process from natural resources is illustrated (**Figure 3**).

However, several factors involving the conversion of a desirable compound into a valuable drug candidate include availability, bioavailability, intellectual property, and the strong pharmacokinetic profile of the compound (268, 290). Sometimes researchers find great bioactivity of a plant-derived compound in *in vitro* analysis but unfortunately, the desired compound becomes ineffectual under *in vivo* conditions (291). *In vivo* is a very crucial step to move to animal trials or subsequent clinical trials. Even if the compound shows promising activity in *in vivo* assay but it can still become ineffective in animal model trials due to a poor pharmacokinetic profile (292). Under *in vivo* condition, the target compound remains in direct contact with cells, while in animal models the compound moves to various stages where it might lose its bioactivity (292). For example, despite curcumin having promising antioxidant, anticancer, anti-inflammatory, and antimicrobial activities, it has not been released as a drug yet due to its poor bioavailability (292). Another propitious drug candidate, epigallocatechin gallate (EGCG), showed antioxidant, antihypertensive, anticancer, antimicrobial, and anti-inflammatory activity (293, 294) but unfortunately, it has also failed to obtain drug designation due to the same reason mentioned for curcumin (292).

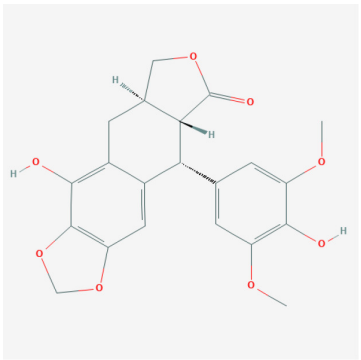
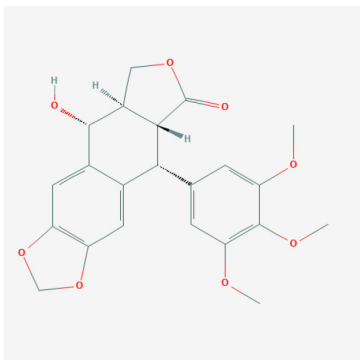
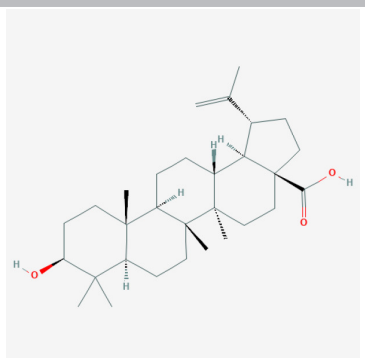
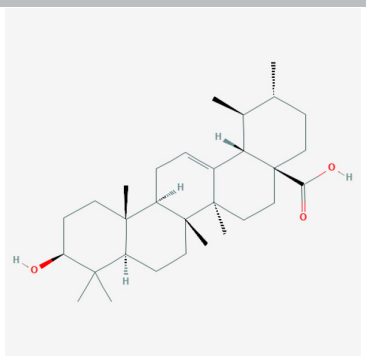
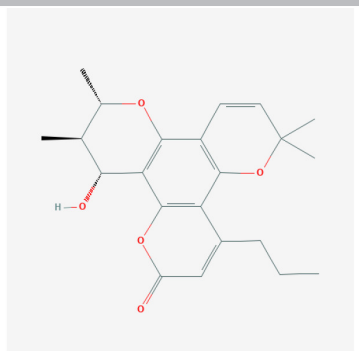
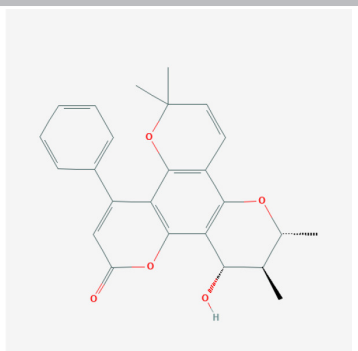
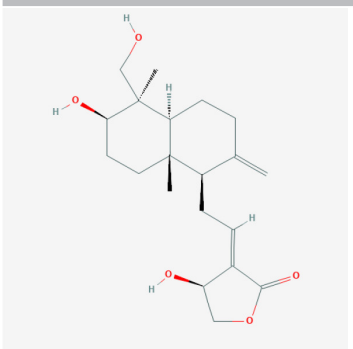
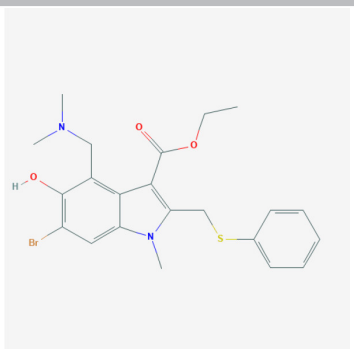
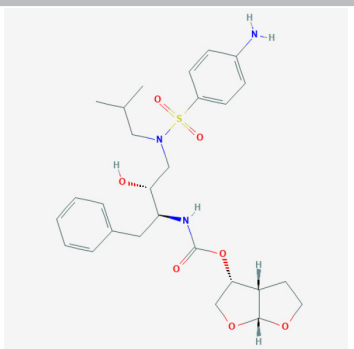
To remedy these problems, researchers around the world are working to develop new approaches. Changing the administration route might increase the bioavailability of a compound. For example, the bioavailability of an anti-inflammatory compound, andrographolide, is increased when it is administered intravenously instead of through oral administration (295). Other methods to enhance the bioavailability of target compound include using drug delivery systems, the nano-formulation of a drug, using adjuvant systems, or altering structural analogs (208, 296). Furthermore, the modification of pharmacokinetic profiles of compounds like absorption, distribution, metabolism, and excretion can escalate its probability as drug candidate (268). Indeed, there is an urgent need for specific protocols for invention of novel bioactive compounds and for this purpose it is very crucial for related organizations, companies, and agencies, including the World Health Organization (WHO), Food and Drug Administration (FDA), European Medicines Agency (EMA), World Trade Organization (WTO), International Conference on Harmonization (ICH), World Intellectual Property Organization (WIPO), biotech companies, pharmaceutical pharmaceuticals companies, and several other companies and agencies, to work together. However, plant-originated therapeutics need to be taken under consideration against SARS-CoV-2 as they have already shown promising hopes for different critical conditions caused by deadly pathogens.

TABLE 5 | Structures of some major PSMs and Drugs used against SARS CoV-2.

ALKALOIDS		
		
Buchapine	Colchicine	Acronine
		
Citrusinine	Rohitukine	
POLYPHENOLS		
		
Aescuflavoside	Diphyllin	Galangin
		
Isoscutellarein	Justicidin	Ternatin

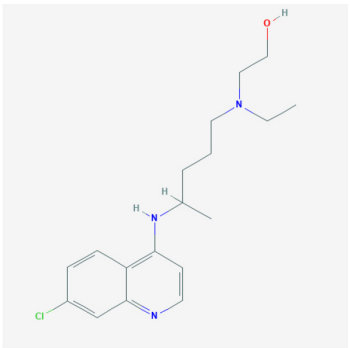
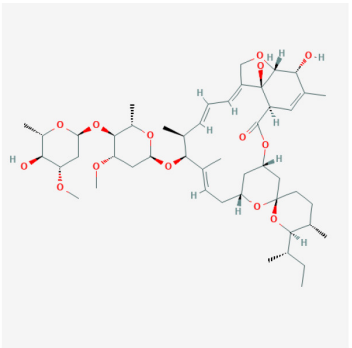
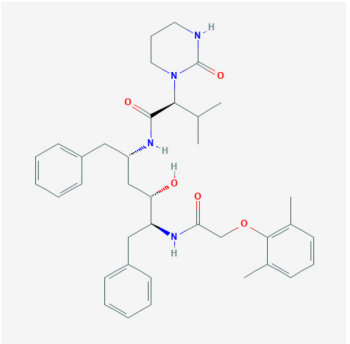
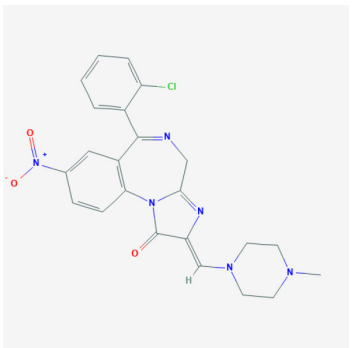
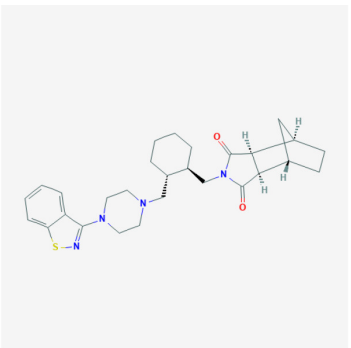
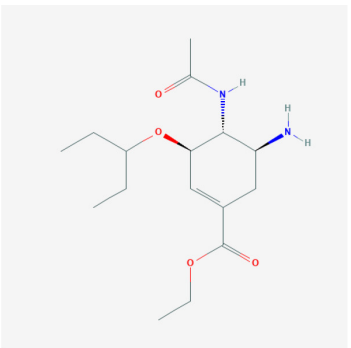
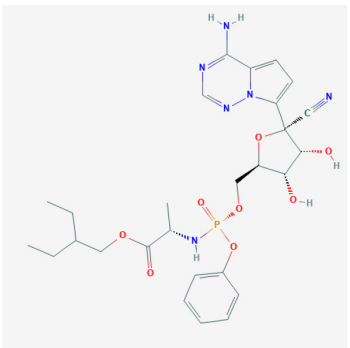
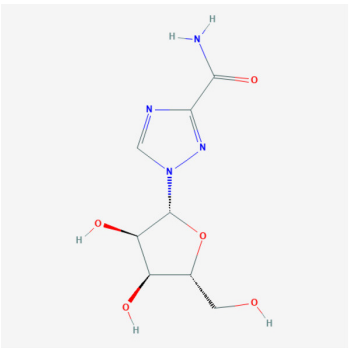
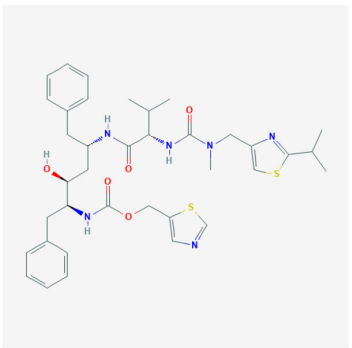
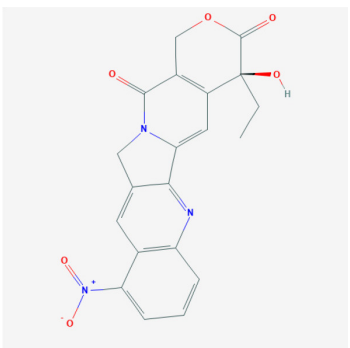
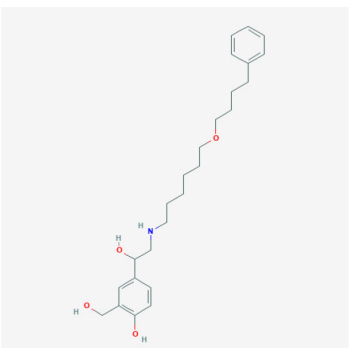
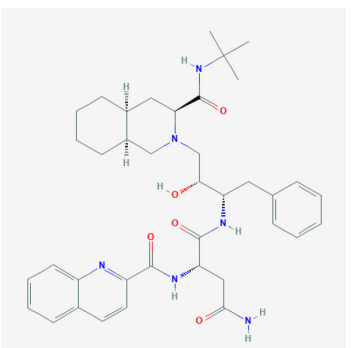
(Continued)

TABLE 5 | Continued

 α-Peltatin	 Podophyllotoxin
TERPENOIDS	
 Betulinic acid	 Ursolic acid
OTHERS	
 -(-)Calanolide	 Inophyllum B
SYNTHETIC DRUGS	
 Andrographolide	 Arbidol
 Darunavir	

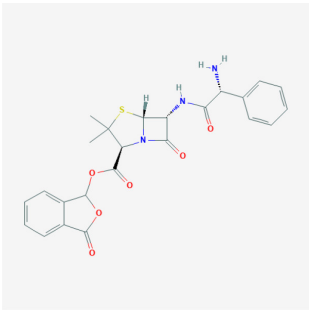
(Continued)

TABLE 5 | Continued

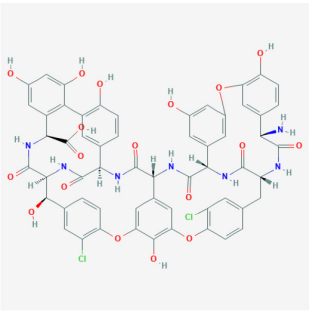
 Hydroxychloroquine	 Ivermectin	 Lopanovir
 Loprazolam	 Lurasidone	 Oseltamivir
 Remdivisir	 Ribavirin	 Ritonavir
 Rubitecan	 Salmeterol	 Saquinavir

(Continued)

TABLE 5 | Continued



Talampicillin



Teicoplanin

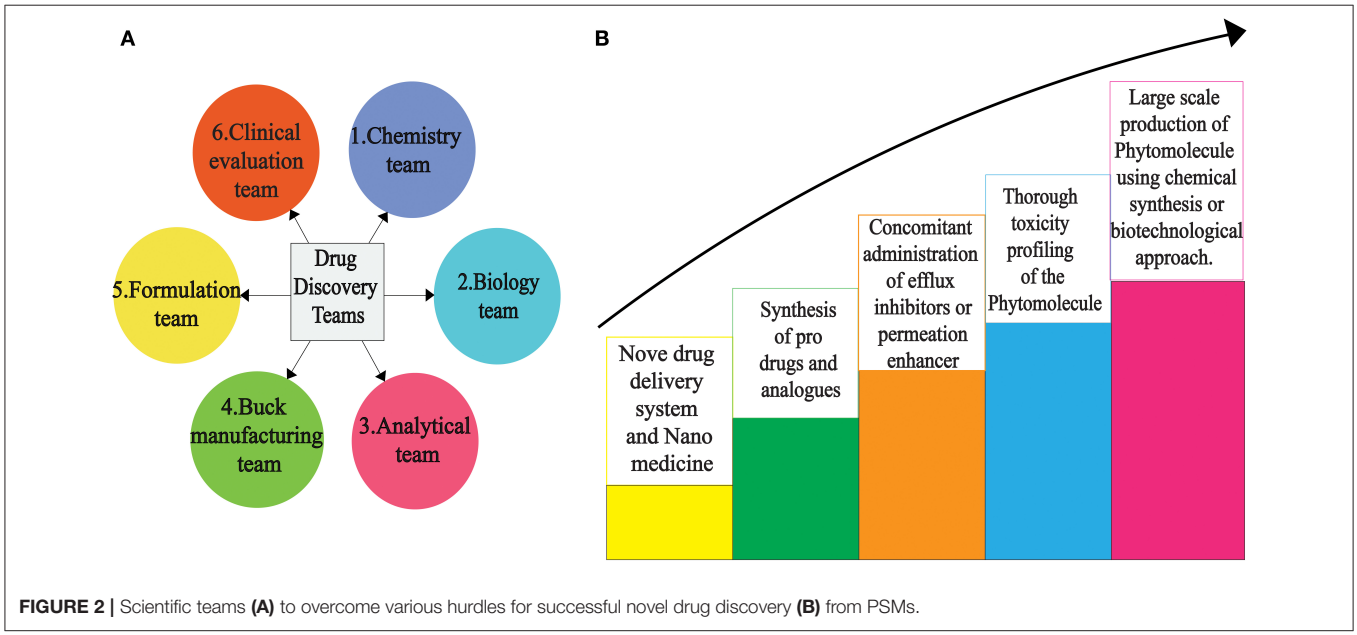


FIGURE 2 | Scientific teams **(A)** to overcome various hurdles for successful novel drug discovery **(B)** from PSMs.

The seven major drug targets of SARS CoV-2 were described before (176). Similarly, screening of PSMs for drug establishment by molecular docking is efficient in terms of time and cost. Even the development of vaccines through computational biology was found to be effective for previous severe viruses like MERS using animal models, target antigens, and probable vaccine candidates (181). But still, there exists a lack of a complete review for PSMs as alternative drug therapeutics. Our review aims at establishing PSMs as a strong and safe candidate for the treatment of SARS CoV-2. Through suggesting probable antiviral plant metabolites or screening, druggability analysis of plant metabolites against SARS-CoV-2 has become a time-saving practice (280, 297). Without establishing a drug development pipeline that includes clinical trials, these suggested candidate PSMs will end up only in journal publications or be shelved as herbal formulations on a supermarket store as a traditional medicine and will never be a modern drug. Undoubtedly, the plant an underutilized source of novel bioactive compounds and is one of the hotspots to fight against this microbial resistance war. The decrypting of PSMs is not increasing so much in comparison to the number of metabolites produced from plants. A biotechnological approach can offer a desired amount of secondary metabolites in a rapid and eco-friendly way against SARS-CoV-2 (298). In addition, plant metabolomics are now used as a tool for discovery of novel drugs from plant resources (299). Characterization of genes and proteins involved in secondary metabolic pathways are also very crucial to understand. Therefore, omics approaches (transcriptomics, proteomics, and metabolomics) have paramount importance in food research and drug discovery (300, 301) for human welfare. Genetic modifications for engineering plant metabolites can be helpful for reaching a specific drug. Quality control of natural products is also very important. So, laboratory support, skilled manpower, and funding is also very important for drug discovery from natural resources.

CONCLUSIONS

Scientists all around the world are trying to discover the most effective antiviral drug to combat SARS CoV-2. In this situation, our study accentuated some plant secondary metabolites that showed prominent antiviral activity against

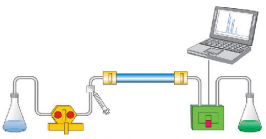
1. Plant selection, Identification, collection and sample preparation
Collection,Herbarium and Authentication of antiviral properties bearing plant by a taxonomist and preparation of sample


Sample Plant

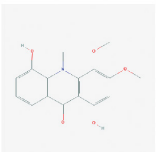
2. Primary screening
Plant crude extract preparation using plant parts (Root, stem, Leaf, flower) Bioassays, Toxicology


Crude preparation

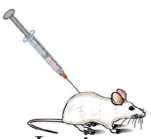
3. Secondary screening
Compound separation, Purification, qualitative and quantitative Analysis, bioassay of pure compound, Toxicology


Purification

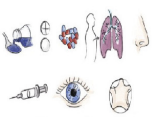
4. Tertiary screening
Structure elucidation, structure modification and bioassay, Toxicology and synthesis


Structure elucidation

5. Preclinical trial
Lead identification, lead optimization and lead development


In vitro

6. Clinical trial
Phase I, Phase II, Phase III trial and treatment review


In vivo

7. Authority Approval
Review of the trial and final approval

FIGURE 3 | Various steps involved in the tedious drug discovery process from plant sources.

coronaviruses through impeding the main machinery used in their pathogenesis and replication cycle. The *in vitro*, *in vivo,* and *in silico* investigations revealed numerous plant-derived compounds with promising anti- SARS CoV and anti- SARS CoV-2 activity [**Table 2**; (179, 220, 222, 233–261, 297)]. Plants are a dramatically underutilized source of bioactive compounds with a broad spectrum of antiviral activities. Some Chinese traditional plant formulations have been reported as being anti-SARS CoV-2 and this formulation is also provided in COVID patients (302, 303). We reported here on 219 plants which act against a wide range of DNA/RNA viruses, but the plant PSMs that showed promising activity against SARS CoV and MERS might be a desired drug candidate against SARS CoV-2. So, this review gathered all antiviral plants in a single platform to facilitate laboratory-based research for the development of novel drug/molecular therapeutics to overcome this and future pandemic situations. The world is facing a serious health crisis, and it needs an effective solution to combat the burning flame of COVID-19. Researchers are trying to find an effective way to overcome this situation, and the present study could help them to think with a new dimension by using the knowledge from the databases based on the plant metabolites (304, 305). Finally, advanced and rapid acting extraction, purification, and characterization techniques used for plant metabolites as well as multidisciplinary expertise and funding are very essential for novel drug discovery.

LITERATURE STUDY

Articles were selected and identified by searching specific keywords and journal citations for each section of a manuscript. Related peer reviewed scientific journal articles were screened from different journal depositories after reviewing abstracts and original data.

AUTHOR'S NOTE

The authors initiated this project to facilitate the research on molecular therapeutics from plant sources as an immediate action in response to the COVID-19 pandemic situation.

AUTHOR CONTRIBUTIONS

FB and MH designed the project. FB prepared the first draft. FB, SH, TR, and MH have investigated the data and completed the manuscript. All authors have read through the manuscript and approved it for submission and publication.

ACKNOWLEDGMENTS

We are thankful to Mr. Md. Fayezur Rahman (MSS in Economics, Comilla University) for drawing the figures as authors expected. Our sincere gratitude to Mr. Mohammad Saifullah (MA in English, University of Chittagong) for proofreading of the manuscript. We are grateful to the editors and reviewers for their valuable comments during the review process to improve the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2020.00444/full#supplementary-material

Supplementary Figure 1 | Different plant families showing antiviral properties. (Each portion of the pie chart describes a specific Family alongside its total number of plants that have antiviral properties).

Supplementary Table 1 | List of secondary metabolites found from medicinal plants.

REFERENCES

1. Brian DA, Baric RS. Coronavirus genome structure and replication. *Curr Top Microbiol Immunol.* (2005) 287:1–30. doi: 10.1007/3-540-26765-4_1
2. Lu R, Zhao X, Li J, Niu P, Yang B, Wu H, et al. Genomic characterisation and epidemiology of 2019 novel coronavirus: implications for virus origins and receptor binding. *Lancet.* (2020) 395:565–74. doi: 10.1016/S0140-6736(20)30251-8
3. Cavanagh D. Coronavirus avian infectious bronchitis virus. *Vet Res.* (2007) 38:281–97. doi: 10.1051/vetres:2006055
4. Ismail MM, Tang Y, Saif YM. Pathogenicity of turkey coronavirus in turkeys and chickens. *Avian Dis.* (2003) 47:515–22. doi: 10.1637/5917
5. Fehr AR, Perlman S. Coronaviruses: an overview of their replication and pathogenesis. *Methods Mol Biol.* (2015) 1282:1–23. doi: 10.1007/978-1-4939-2438-7_1
6. Lan, Jun, Jiwan Ge, Jinfang Yu, Sisi Shan, Huan Zhou, et al. Structure of the SARS-CoV-2 spike receptor-binding domain bound to the ACE2 receptor. *Nature*. (2020) 581:215–20 doi: 10.1101/2020.02.19.956235
7. Chen Y, Peng H, Wang L, Zhao Y, Zeng L, Gao H, et al. Infants born to mothers with a new coronavirus (COVID-19). *Front Pediatr.* (2020) 8:104. doi: 10.3389/fped.2020.00104
8. WHO. *Director-General's Opening Remarks at the Media Briefing on COVID-19*. (2020). Available online at: https://www.who.int/dg/speeches/detail/who-director-general-s-opening-remarks-at-the-media-briefing-on-covid-19 (accessed May 15, 2020).
9. WHO. *Director-General's Opening Remarks at the Media Briefing on COVID-19.* (2020). Available online at: https://www.who.int/dg/speeches/detail/who-director-general-sopening-remarks-at-the-media-briefing-on-covid-19 (accessed April 5, 2020).
10. Kanne JP. Chest CT findings in 2019 novel coronavirus (2019-nCoV) infections from Wuhan, China: Key points for the radiologist. *Radiology.* (2020) 295:16–7. doi: 10.1148/radiol.2020200241
11. Zumla A, Chan JFW, Azhar EI, Hui DSC, Yuen KY. Coronaviruses-drug discovery and therapeutic options. *Nat Rev Drug Discov.* (2016) 15:327–47. doi: 10.1038/nrd.2015.37
12. Heymann DL, Rodier G. Global surveillance, national surveillance, and SARS. *Emerg Infect Dis.* (2004) 10:173–5. doi: 10.3201/eid1002.031038
13. Zaki AM, Van Boheemen S, Bestebroer TM, Osterhaus ADME, Fouchier RAM. Isolation of a novel coronavirus from a man with pneumonia in Saudi Arabia. *N Engl J Med.* (2012) 367:1814–20. doi: 10.1056/NEJMoa1211721
14. Shetty R, Ghosh A, Honavar SG, Khamar P, Sethu S. Therapeutic opportunities to manage COVID-19/SARS-CoV-2 infection: present and future. *Indian J Ophthalmol.* (2020) 68:693–702. doi: 10.4103/ijo.IJO_639_20
15. Hirano T, Murakami M. COVID-19: a new virus, but a familiar receptor and cytokine release syndrome. *Immunity.* (2020) 52:731–3. doi: 10.1016/j.immuni.2020.04.003

16. Pedersen SF, Ho YC. SARS-CoV-2: a storm is raging. *J Clin Invest.* (2020) 130:2202–5. doi: 10.1172/JCI137647
17. Coperchini F, Chiovato L, Croce L, Magri F, Rotondi M. The cytokine storm in COVID-19: an overview of the involvement of the chemokine/chemokine-receptor system. *Cytokine Growth Factor Rev.* (2020) 53:25–32. doi: 10.1016/j.cytogfr.2020.05.003
18. Moses T, Goossens A. Plants for human health: greening biotechnology and synthetic biology. *J Exp Bot.* (2017) 68:4009–11. doi: 10.1093/jxb/erx268
19. Schaal B. Plants and people: our shared history and future. *Plants People Planet.* (2019) 1:14–9. doi: 10.1002/ppp3.12
20. World Health Organization. *WHO Global Report on Traditional and Complementary Medicine 2019*. World Health Organization (2019).
21. Hoque Haque MI, Chowdhury ABMA, Shahjahan M, Harun MGD. Traditional healing practices in rural Bangladesh: a qualitative investigation. *BMC Complement Altern Med.* (2018) 18:62. doi: 10.1186/s12906-018-2129-5
22. Ozioma E, Okaka A. Herbal medicines in African traditional medicine. In: Ozioma EJ and Chinwe OAN, editors. *Herbal Medicine.* Intratech open (2019). p. 1–25.
23. Jassim SAA, Naji MA. Novel antiviral agents: a medicinal plant perspective. *J Appl Microbiol.* (2003) 95:412–27. doi: 10.1046/j.1365-2672.2003.02026.x
24. Hussain W, Haleem KS, Khan I, Tauseef I, Qayyum S, Ahmed B, et al. Medicinal plants: a repository of antiviral metabolites. *Future Virol.* (2017) 12:299–308. doi: 10.2217/fvl-2016-0110
25. Özçelik B, Kartal M, Orhan I. Cytotoxicity, antiviral and antimicrobial activities of alkaloids, flavonoids, and phenolic acids. *Pharm Biol.* (2011) 49:396–402. doi: 10.3109/13880209.2010.519390
26. Pick A, Ling K, Khoo BF, Seah CH, Foo KY, Cheah RK. Inhibitory activities of methanol extracts of Andrographis Paniculata and Ocimum Sanctum against Dengue-1 virus. In: *International Conference on Biological, Environment & Food Engineering*. Bali (2014). p. 6.
27. Namazi R, Zabihollahi R, Behbahani M, Rezae A. Inhibitory activity of *Avicennia marina*, a medicinal plant in persian folk medicine, against HIV and HSV. *Iran J Pharm Res.*. (2013) 12:435–43.
28. Zhou B, Yang Z, Feng Q, Liang X, Li J, Zanin M, et al. Aurantiamide acetate from *baphicacanthus cusia* root exhibits anti-inflammatory and anti-viral effects via inhibition of the NF-κB signaling pathway in influenza A virus-infected cells. *J Ethnopharmacol.* (2017) 199:60–7. doi: 10.1016/j.jep.2017.01.038
29. Castillo-Maldonado I, Moreno-Altamirano MMB, Serrano-Gallardo LB. Anti-dengue serotype-2 activity effect of *Sambucus nigra* leaves-and flowers-derived compounds. *Virol Res Rev.* (2017) 1:1–5. doi: 10.15761/VRR.1000117
30. Chen C, Zuckerman DM, Brantley S, Sharpe M, Childress K, Hoiczyk E, et al. *Sambucus nigra* extracts inhibit infectious bronchitis virus at an early point during replication. *BMC Vet Res.* (2014) 10:24. doi: 10.1186/1746-6148-10-24
31. Andleeb R, Ashraf A, Muzammil S, Naz S, Asad F, Ali T, et al. Analysis of bioactive composites and antiviral activity of *Iresine herbstii* extracts against Newcastle disease virus in ovo. *Saudi J Biol Sci.* (2020) 27:335–40. doi: 10.1016/j.sjbs.2019.10.002
32. Szlµvik L, Minµrovits J, Forgo P. Alkaloids from *Leucojum vernum* and antiretroviral activity of *Amaryllidaceae* alkaloids. *Planta Medica.* (2004) 70:871–3. doi: 10.1055/s-2004-827239
33. Lopes RC, Oliveira DB, Costa SS, Miranda MMFS, Romanos MT V, Santos NSO, et al. *In vitro* anti-rotavirus activity of some medicinal plants used in Brazil against diarrhea. *J Ethnopharmacol.* (2005) 99:403–7. doi: 10.1016/j.jep.2005.01.032
34. Reichling J, Neuner A, Sharaf M, Harkenthal M, Schnitzler P. Antiviral activity of *Rhus aromatica* (fragrant sumac) extract against two types of herpes simplex viruses in cell culture. *Pharmazie.* (2009) 64:538–41.
35. Modi M, Nutan, Pancholi B, Kulshrestha S, Rawat AKS, Malhotra S, et al. Anti-HIV-1 activity, protease inhibition and safety profile of extracts prepared from *Rhus parviflora*. *BMC Complement Altern Med.* (2013) 13:158. doi: 10.1186/1472-6882-13-158
36. Nocchi SR, Companhoni MVP, De Mello JCP, Dias Filho BP, Nakamura CV, Carollo CA, et al. Antiviral activity of crude hydroethanolic extract from *Schinus terebinthifolia* against herpes simplex virus type 1. *Planta Med.* (2017) 83:509–18. doi: 10.1055/s-0042-117774
37. Park JY, Ko JA, Kim DW, Kim YM, Kwon HJ, Jeong HJ, et al. Chalcones isolated from *Angelica keiskei* inhibit cysteine proteases of SARS-CoV. *J Enzyme Inhib Med Chem.* (2016) 31:23–30. doi: 10.3109/14756366.2014.1003215
38. Tolo FM, Rukunga GM, Muli FW, Njagi ENM, Njue W, Kumon K, et al. Anti-viral activity of the extracts of a Kenyan medicinal plant *Carissa edulis* against Herpes simplex virus. *J Ethnopharmacol.* (2006) 104:92–9. doi: 10.1016/j.jep.2005.08.053
39. Bonvicini F, Lianza M, Mandrone M, Poli F, Gentilomi GA, Antognoni F. *Hemidesmus indicus* (L.) R. Br. Extract inhibits the early step of herpes simplex type 1 and type 2 replication. *New Microbiol.* (2018) 41:187–94.
40. Rittà M, Marengo A, Civra A, Lembo D, Cagliero C, Kant K, et al. Antiviral activity of a *Arisaema tortuosum* leaf extract and some of its constituents against herpes simplex virus type 2. *Planta Med.* (2020) 86:267–75. doi: 10.1055/a-1087-8303
41. Lee JS, Ko E, Hwang HYESUK, Lee Y, Kwon Y, Kim M, et al. Antiviral activity of ginseng extract against respiratory syncytial virus infection. *Int J Mol Med.* (2014) 34:183–90. doi: 10.3892/ijmm.2014.1750
42. Lee MH, Lee B, Jung J, Cheon D, Kim K, Choi C. Antiviral effect of Korean red ginseng extract and ginsenosides on murine norovirus and feline calicivirus as surrogates for human norovirus. *J Ginseng Res.* (2011) 35:429–35. doi: 10.5142/jgr.2011.35.4.429
43. Choi J, Jin Y, Lee H, Oh TW, Yim N, Cho W. Protective effect of *Panax notoginseng* root water extract against influenza a virus infection by enhancing antiviral interferon-mediated immune responses and natural killer cell activity. *Front Immunol.* (2017) 8:1542. doi: 10.3389/fimmu.2017.01542
44. Glatthaar-Saalmüller B, Fal AM, Schönknecht K, Conrad F, Sievers H, Saalmüller A. Antiviral activity of an aqueous extract derived from *Aloe arborescens Mill.* against a broad panel of viruses causing infections of the upper respiratory tract. *Phytomedicine.* (2015) 22:911–20. doi: 10.1016/j.phymed.2015.06.006
45. Zandi K, Zadeh MA, Sartavi K, Rastian Z. Antiviral activity of *Aloe vera* against herpes simplex virus type 2 : an *in vitro* study. *Afr J Biotechnol.* (2007) 6:1770203. doi: 10.5897/AJB2007.000-2276
46. Moradi M, Rafieian-kopaei M, Karimi A. A review study on the effect of Iranian herbal medicines against *in vitro* replication of herpes simplex virus. *Avicenna J Phytomed.* (2016) 6:506–15.
47. Soltan MM, Zaki AK. Antiviral screening of forty-two Egyptian medicinal plants. *J Ethnopharmacol.* (2009) 126:102–7. doi: 10.1016/j.jep.2009.08.001
48. Patocka J, Navratilova Z. *Achillea fragrantissima* : pharmacology review. *Clin Oncol.* (2019) 4:1601.
49. Visintini M, Redko F, Muschietti L, Campos R, Martino V, Cavallaro LV. *In vitro* antiviral activity of plant extracts from Asteraceae medicinal plants. *Virol J.* (2013) 10:245. doi: 10.1186/1743-422X-10-245
50. Derksen A, Kühn J, Hafezi W, Sendker J, Ehrhardt C, Ludwig S, et al. Antiviral activity of hydroalcoholic extract from *Eupatorium perfoliatum* L. against the attachment of influenza A virus. *J Ethnopharmacol.* (2016) 188:144–52. doi: 10.1016/j.jep.2016.05.016
51. Lani R, Hassandarvish P, Chiam CW, Moghaddam E, Chu JJH, Rausalu K, et al. Antiviral activity of *silymarin* against chikungunya virus. *Sci Rep*. (2015) 5:11421. doi: 10.1038/srep11421
52. Benassi-Zanqueta É, Marques CF, Valone LM, Pellegrini BL, Bauermeister A, Ferreira ICP, et al. Evaluation of anti-HSV-1 activity and toxicity of hydroethanolic extract of *Tanacetum parthenium* (L.) Sch.Bip. (Asteraceae). *Phytomedicine.* (2019) 55:249–54. doi: 10.1016/j.phymed.2018.06.040
53. Rehman S, Ijaz B, Fatima N, Aun S. Sciencedirect therapeutic potential of *Taraxacum officinale* against HCV NS5B polymerase : *in-vitro* and *in silico* study. *Biomed Pharmacother.* (2016) 83:881–91. doi: 10.1016/j.biopha.2016.08.002
54. He W, Han H, Wang W, Gao B. Anti-influenza virus effect of aqueous extracts from dandelion. *Virol J.* (2011) 8:538. doi: 10.1186/1743-422X-8-538
55. Rothan HA, Zulqarnain M, Ammar YA, Tan EC, Rahman NA, Yusof R. Screening of antiviral activities in medicinal plants extracts against dengue virus using dengue NS2B-NS3 protease assay. *Trop Biomed.* (2014) 31:286–96.
56. Mgole S, Pieters L, David O, Apers S, Vingerhoets R, Cos P, et al. Screening of some Tanzanian medicinal plants from Bunda District for

antibacterial, antifungal and antiviral activities. *J Ethnopharmacol.* (2008) 119:58–66. doi: 10.1016/j.jep.2008.05.033
57. Al-thobaiti SA, Zeid IMA. Phytochemistry and pharmaceutical evaluation of *Balanites aegyptiaca* : an overview. *J Exp Biol Agric Sci.* (2018) 6:453–65 doi: 10.18006/2018.6(3).453.465
58. Cho WK, Kim H, Choi YJ, Yim NH, Yang HJ, Ma JY. *Epimedium koreanum* Nakai water extract exhibits antiviral activity against porcine epidermic diarrhea virus *in vitro* and *in vivo*. *Evid Based Complement Altern Med.* (2012) 2012:985150. doi: 10.1155/2012/985151
59. Park JY, Jeong HJ, Kim JH, Kim YM, Park SJ, Kim D, et al. Diarylheptanoids from *Alnus japonica* inhibit papain-like protease of severe acute respiratory syndrome coronavirus. *Biol Pharm Bull.* (2012) 35:2036–42. doi: 10.1248/bpb.b12-00623
60. Tung NH, Kwon HJ, Kim JH, Ra JC, Ding Y, Kim JA, et al. Anti-influenza diarylheptanoids from the bark of *Alnus japonica*. *Bioorganic Med Chem Lett.* (2010) 20:1000–3. doi: 10.1016/j.bmcl.2009.12.057
61. Lin CW, Tsai FJ, Tsai CH, Lai CC, Wan L, Ho TY, et al. Anti-SARS coronavirus 3C-like protease effects of *Isatis indigotica* root and plant-derived phenolic compounds. *Antiviral Res.* (2005) 68:36–42. doi: 10.1016/j.antiviral.2005.07.002
62. Chen F, Yang L, Huang Y, Chen Y, Sang H, Duan W, et al. Isocorilagin, isolated from *Canarium album (Lour.)* Raeusch, as a potent neuraminidase inhibitor against influenza A virus. *Biochem Biophys Res Commun.* (2020) 523:183–9. doi: 10.1016/j.bbrc.2019.12.043
63. César GZJ, Alfonso MGG, Marius MM, Elizabeth EM, Ángel CBM, Maira HR, Guadalupe CLM, et al. Inhibition of HIV-1 reverse transcriptase, toxicological and chemical profile of *Calophyllum brasiliense* extracts from Chiapas, Mexico. *Fitoterapia.* (2011) 82:1027–34. doi: 10.1016/j.fitote.2011.06.006
64. Ibrahim AK, Youssef AI, Arafa AS, Ahmed SA. Anti-H5N1 virus flavonoids from *Capparis sinaica* Veill. *Nat Prod Res.* (2013) 27:2149–53. doi: 10.1080/14786419.2013.790027
65. Ghazal EA, Khamis IMA, Elhaw MHM. Chemical constituents of *Capparis sinaica* Veill. plant and its antimicrobial effects. *Middle East J Appl Sci.* (2015) 5:411–22.
66. Lam S-Z, Ng T-B. A protein with antiproliferative, antifungal and HIV-1 reverse transcriptase inhibitory activities from caper (*Capparis spinosa*) seeds. *Phytomedicine.* (2009) 16:444–50. doi: 10.1016/j.phymed.2008.09.006
67. Callies O, Bedoya LM, Beltra M, Mun A, Obrego P, Osorio AA, et al. Isolation, structural modification, and HIV inhibition of pentacyclic lupane-type triterpenoids from *Cassine xylocarpa* and *Maytenus cuzcoina*. *J Nat Prod.* (2015) 78:1045–55. doi: 10.1021/np501025r
68. Inoue R. Orally administered *Salacia reticulata* extract reduces h1n1 influenza clinical symptoms in murine lung tissues putatively due to enhanced natural killer cell activity. *Front Immunol.* (2016) 7:115. doi: 10.3389/fimmu.2016.00115
69. Rebensburg S, Helfer M, Schneider M, Koppensteiner H, Eberle J, Schindler M, et al. Potent *in vitro* antiviral activity of *Cistus incanus* extract against HIV and Filoviruses targets viral envelope proteins. *Sci Rep.* (2016) 6:20394. doi: 10.1038/srep20394
70. Javed T, Ashfaq UA, Riaz S, Rehman S, Riazuddin S. In-vitro antiviral activity of *Solanum nigrum* against Hepatitis C Virus. *Virol J.* (2011) 8:26. doi: 10.1186/1743-422X-8-26
71. Alcami J, Bermejo P. Phytomedicine Ellagitannins from *Tuberaria lignosa* as entry inhibitors of HIV. *Phytomedicine.* (2010) 17:69–74. doi: 10.1016/j.phymed.2009.08.008
72. Mushi NF, Mbwambo ZH, Innocent E, Tewtrakul S. Activities of aqueous ethanolic extracts from *Combretum adenogonium* Steud. Ex A. Rich *(Combretaceae)*. *BMC Complement Altern Med.* (2012) 12:163. doi: 10.1186/1472-6882-12-163
73. Liu M, Katerere DR, Gray AI, Seidel V. Phytochemical and antifungal studies on *Terminalia mollis* and *Terminalia brachystemma*. *Fitoterapia.* (2009) 80:369–73. doi: 10.1016/j.fitote.2009.05.006
74. Lavoie S, Côté I, Pichette A, Gauthier C, Ouellet M, Nagau-Lavoie F, et al. Chemical composition and anti-herpes simplex virus type 1 (HSV-1) activity of extracts from *Cornus canadensis*. *BMC Complement Altern Med.* (2017) 17:2. doi: 10.1186/s12906-017-1618-2
75. Hsieh CF, Chen YL, Lin CF, Ho JY, Huang CH, Chiu CH, et al. An extract from *Taxodium distichum* targets hemagglutinin- and neuraminidase-related activities of influenza virus *in vitro*. *Sci Rep.* (2016) 6:36015. doi: 10.1038/srep36015
76. Xu H, Ma Y, Huang X, Geng C, Wang H. Bioactivity-guided isolation of anti-hepatitis B virus active sesquiterpenoids from the traditional Chinese medicine : Rhizomes of *Cyperus rotundus*. *J Ethnopharmacol.* (2015) 171:131–40. doi: 10.1016/j.jep.2015.05.040
77. Danciu C, Muntean D, Alexa E, Farcas C, Oprean C, Zupko I, et al. Phytochemical characterization and evaluation of the antimicrobial, antiproliferative and pro-apoptotic potential of *Ephedra alata* Decne. hydroalcoholic extract against the MCF-7 breast cancer cell line. *Molecules.* (2018) 24:13. doi: 10.3390/molecules24010013
78. Churqui MP, Lind L, Thörn K, Svensson A, Savolainen O, Aranda KT, et al. Extracts of *Equisetum giganteum* L and *Copaifera reticulate* Ducke show strong antiviral activity against the sexually transmitted pathogen herpes simplex virus type 2. *J Ethnopharmacol.* (2018) 210:192–7. doi: 10.1016/j.jep.2017.08.010
79. Shamsabadipour S, Ghanadian M, Saeedi H, Reza Rahimnejad M, Mohammadi-Kamalabadi M, Ayatollahi SM, et al. Triterpenes and steroids from *Euphorbia denticulata* Lam. with anti-herpes symplex virus activity. *Iran J Pharm Res.* (2013) 12:759–67.
80. Gyuris A, Szlávik L, Minárovits J, Vasas A, Molnár J, Hohmann J. Antiviral activities of extracts of *Euphorbia hirta* L. against HIV-1, HIV-2 and SIVmac251. *In Vivo.* (2008) 23:429–32.
81. Jiang C, Luo P, Zhao Y, Hong J, Morris-Natschke SL, Xu J, et al. Carolignans from the aerial parts of *Euphorbia sikkimensis* and their anti-HIV activity. *J Nat Prod.* (2016) 79:578–83. doi: 10.1021/acs.jnatprod.5b01012
82. Shoji M, Woo SY, Masuda A, Win NN, Ngwe H, Takahashi E, et al. Anti-influenza virus activity of extracts from the stems of *Jatropha multifida* Linn. Collected in Myanmar. *BMC Complement Altern Med.* (2017) 17:76. doi: 10.1186/s12906-017-1612-8
83. Ghoke SS, Sood R, Kumar N, Pateriya AK, Bhatia S, Mishra A, et al. Evaluation of antiviral activity of *Ocimum sanctum* and *Acacia arabica* leaves extracts against H9N2 virus using embryonated chicken egg model. *BMC Complement Altern Med.* (2018) 18:174. doi: 10.1186/s12906-018-2238-1
84. Makau JN, Watanabe K, Mohammed MMD, Nishida N. Antiviral activity of peanut (*Arachis hypogaea* L.) skin extract against human influenza viruses. *J Med Food.* (2018) 21:777–84. doi: 10.1089/jmf.2017.4121
85. Knipping K, Garssen J, van't Land B. An evaluation of the inhibitory effects against rotavirus infection of edible plant extracts. *Virol J.* (2012) 9:137. doi: 10.1186/1743-422X-9-137
86. Fahmy NM, Al-Sayed E, Moghannem S, Azam F, El-Shazly M, Singab AN. Breaking down the barriers to a natural antiviral agent: antiviral activity and molecular docking of *Erythrina speciosa* extract, fractions, and the major compound. *Chem Biodivers.* (2020) 17:2. doi: 10.1002/cbdv.201900511
87. Donalisio M, Cagno V, Civra A, Gibellini D, Musumeci G, Rittà M, et al. The traditional use of *Vachellia nilotica* for sexually transmitted diseases is substantiated by the antiviral activity of its bark extract against sexually transmitted viruses. *J Ethnopharmacol.* (2018) 213:403–8. doi: 10.1016/j.jep.2017.11.039
88. Nutan SK, Modi M, Dezzutti CS, Kulshreshtha S, Rawat AKS, Srivastava SK, et al. Extracts from *Acacia catechu* suppress HIV-1 replication by inhibiting the activities of the viral protease and Tat. *Virol J.* (2013) 10:309. doi: 10.1186/1743-422X-10-309
89. Karimi A, Rafieian-kopaei M, Moradi M. Anti -herpes simplex virus type-1 activity and phenolic content of crude ethanol extract and four corresponding fractions of *Quercus brantii* L Acorn. *J Evid Based Complement Altern Med.* (2016) 22:455–61. doi: 10.1177/2156587216676421
90. Rafieian-kopaei M, Saeedi M, Asgari S, Karimi A, Moradi M-T. Antiviral activity of *Quercus persica* L.: high efficacy and low toxicity. *Adv Biomed Res.* (2013) 2:36. doi: 10.4103/2277-9175.109722
91. Choi JG, Kim YS, Kim JH, Chung HS. Antiviral activity of ethanol extract of *Geranii Herba* and its components against influenza viruses via neuraminidase inhibition. *Sci Rep.* (2019) 9:12132. doi: 10.1038/s41598-019-48430-8
92. Helfer M, Koppensteiner H, Schneider M, Rebensburg S, Forcisi S, Müller C, et al. The root extract of the medicinal plant *Pelargonium*

sidoides is a potent HIV-1 attachment inhibitor. *PLoS ONE.* (2014) 9:e87487. doi: 10.1371/journal.pone.0087487
93. Michaelis M, Doerr HW, Cinatl J. Investigation of the influence of EPs® 7630, a herbal drug preparation from *Pelargonium sidoides*, on replication of a broad panel of respiratory viruses. *Phytomedicine.* (2011) 18:384–6. doi: 10.1016/j.phymed.2010.09.008
94. Haasbach E, Hartmayer C, Hettler A, Sarnecka A, Wulle U, Ehrhardt C, et al. Antiviral activity of Ladania067, an extract from wild black currant leaves against influenza a virus *in vitro* and *in vivo*. *Front Microbiol.* (2014) 5:171. doi: 10.3389/fmicb.2014.00171
95. Theisen LL, Erdelmeier CAJ, Spoden GA, Boukhallouk F, Sausy A, Florin L, et al. Tannins from *Hamamelis virginiana* bark extract: characterization and improvement of the antiviral efficacy against influenza, a virus and human papillomavirus. *PLoS ONE.* (2014) 9:e88062. doi: 10.1371/journal.pone.0088062
96. Erdelmeier CAJ, Cinatl J, Rabenau H, Doerr HW, Biber A, Koch E. Antiviral and antiphlogistic activities of *Hamamelis virginiana* bark. *Planta Med.* (1996) 62:241–5. doi: 10.1055/s-2006-957868
97. Chen SG, Leu YL, Cheng ML, Ting SC, Liu CC, Wang S Der, et al. Anti-enterovirus 71 activities of *Melissa officinalis* extract and its biologically active constituent rosmarinic acid. *Sci Rep.* (2017) 7:12264. doi: 10.1038/s41598-017-12388-2
98. Schnitzler P, Schuhmacher A, Astani A, Reichling J. *Melissa officinalis* oil affects infectivity of enveloped herpesviruses. *Phytomedicine.* (2008) 15:734–40. doi: 10.1016/j.phymed.2008.04.018
99. Tang LIC, Ling APK, Koh RY, Chye SM, Voon KGL. Screening of anti-dengue activity in methanolic extracts of medicinal plants. *BMC Complement Altern Med.* (2012) 12:3. doi: 10.1186/1472-6882-12-3
100. Chiu LCM, Zhu W, Ooi VEC. A polysaccharide fraction from medicinal herb *Prunella vulgaris* downregulates the expression of herpes simplex virus antigen in Vero cells. *J Ethnopharmacol.* (2004) 93:63–8. doi: 10.1016/j.jep.2004.03.024
101. Brindley MA, Widrlechner MP, McCoy JA, Murphy P, Hauck C, Rizshsky L, et al. Inhibition of lentivirus replication by aqueous extracts of *Prunella vulgaris*. *Virol J.* (2009) 6:8. doi: 10.1186/1743-422X-6-8
102. Ma F-W, Kong S-Y, Tan H-S, Wu R, Xia B, Zhou Y, et al. Structural characterization and antiviral effect of a novel polysaccharide PSP-2B from *Prunellae Spica*. *Carbohydr Polym.* (2016) 152:699–709. doi: 10.1016/j.carbpol.2016.07.062
103. Mancini DAP, Torres RP, Pinto JR, Mancini-Filho J. Inhibition of DNA virus: Herpes-1 (HSV-1) in cellular culture replication, through an antioxidant treatment extracted from *rosemary* spice. *Brazilian J Pharm Sci.* (2009) 45:127–33. doi: 10.1590/S1984-82502009000100016
104. Shi H, Ren K, Lv B, Zhang W, Zhao Y, Tan RX, et al. Baicalin from *Scutellaria baicalensis* blocks respiratory syncytial virus (RSV) infection and reduces inflammatory cell infiltration and lung injury in mice. *Sci Rep.* (2016) 6:35851. doi: 10.1038/srep35851
105. Cao YG, Hao Y, Li ZH, Liu ST, Wang LX. Antiviral activity of polysaccharide extract from *Laminaria japonica* against respiratory syncytial virus. *Biomed Pharmacother.* (2016) 84:1705–10. doi: 10.1016/j.biopha.2016.10.082
106. Yarmolinsky L, Zaccai M, Ben-shabat S, Mills D, Huleihel M. Antiviral activity of ethanol extracts of *Ficus binjamina* and *Lilium candidum in vitro*. (2009) 26:307–13. doi: 10.1016/j.nbt.2009.08.005
107. Tsai YC, Hohmann J, El-Shazly M, Chang LK, Dankó B, Kúsz N, et al. Bioactive constituents of *Lindernia crustacea* and its anti-EBV effect via Rta expression inhibition in the viral lytic cycle. *J Ethnopharmacol.* (2020) 250:112493. doi: 10.1016/j.jep.2019.112493
108. Boff L, Silva IT, Argenta DF, Farias LM, Alvarenga LF, Pádua RM, et al. *Strychnos pseudoquina* A. St. Hil.: a Brazilian medicinal plant with promising *in vitro* antiherpes activity. *J Appl Microbiol.* (2016) 121:1519–29. doi: 10.1111/jam.13279
109. Arunkumar J, Rajarajan S. Study on antiviral activities, drug-likeness and molecular docking of bioactive compounds of *Punica granatum* L. to Herpes simplex virus - 2 (HSV-2). *Microb Pathog.* (2018) 118:301–9. doi: 10.1016/j.micpath.2018.03.052
110. Haidari M, Ali M, Ward Casscells S, Madjid M. Pomegranate (*Punica granatum*) purified polyphenol extract inhibits influenza virus and has a synergistic effect with oseltamivir. *Phytomedicine.* (2009) 16:1127–36. doi: 10.1016/j.phymed.2009.06.002
111. Fang C, Chen S, Wu H, Ping Y, Lin C. Honokiol, a Lignan biphenol derived from the magnolia tree, inhibits Dengue virus type 2 infection. *Viruses.* (2015) 7:4894–910. doi: 10.3390/v7092852
112. Baatartsogt T, Bui VN, Trinh DQ, Yamaguchi E, Gronsang D, Thampaisarn R, et al. High antiviral effects of hibiscus tea extract on the H5 subtypes of low and highly pathogenic avian influenza viruses. *J Vet Med Sci.* (2016) 78:1405–11. doi: 10.1292/jvms.16-0124
113. Sood R, Raut R, Tyagi P, Pareek PK, Barman TK, Singhal S, et al. *Cissampelos pareira* Linn: Natural source of potent antiviral activity against all four Dengue virus serotypes. *PLoS Negl Trop Dis.* (2015) 9:e0004255. doi: 10.1371/journal.pntd.0004255
114. Yarmolinsky L, Huleihel M, Zaccai M, Ben-shabat S. Potent antiviral flavone glycosides from *Ficus benjamina* leaves. *Fitoterapia.* (2012) 83:362–7. doi: 10.1016/j.fitote.2011.11.014
115. Aref HL, Gaaliche B, Fekih A. *In vitro* cytotoxic and antiviral activities of *Ficus carica* latex extracts. *Nat Prod Res.* (2011) 25:310–9. doi: 10.1080/14786419.2010.528758
116. Ghosh M, Civra A, Rittà M, Cagno V, Mavuduru SG, Awasthi P, et al. *Ficus religiosa* L. bark extracts inhibit infection by herpes simplex virus type 2 *in vitro*. *Arch Virol.* (2016) 161:3509–14. doi: 10.1007/s00705-016-3032-3
117. Huang NC, Hung WT, Tsai WL, Lai FY, Lin YS, Huang MS, et al. *Ficus septica* plant extracts for treating Dengue virus *in vitro*. *Peer J.* (2017) 5:e3448. doi: 10.7717/peerj.3448
118. Sa S, Abdullahi H. Antifungal activity and phytochemical analysis of *Ficus sycomorus* leaf extract on malassezia glubosa. *Adv Plants Agric Res.* (2018) 8:432–6. doi: 10.15406/apar.2018.08.00362
119. Batiha GES, Alkazmi LM, Wasef LG, Beshbishy AM, Nadwa EH, Rashwan EK. *Syzygium aromaticum* L. (myrtaceae): traditional uses, bioactive chemical constituents, pharmacological and toxicological activities. *Biomolecules.* (2020) 10:202. doi: 10.3390/biom10020202
120. Benzekri R, Bouslama L, Papetti A, Hammami M, Smaoui A, Limam F. Anti HSV-2 activity of *Peganum harmala* (L.) and isolation of the active compound. *Microb Pathog.* (2018) 114:291–8. doi: 10.1016/j.micpath.2017.12.017
121. Li J, Yang X, Huang L. Anti-Influenza virus activity and constituents characterization of *Paeonia delavayi* extracts. *Molecules.* (2016) 21:1133. doi: 10.3390/molecules21091133
122. Ho JY, Chang HW, Lin CF, Liu CJ, Hsieh CF, Horng JT. Characterization of the anti-influenza activity of the Chinese herbal plant *Paeonia lactiflora*. *Viruses.* (2014) 6:1861–75. doi: 10.3390/v6041861
123. Cho JK, Curtis-Long MJ, Lee KH, Kim DW, Ryu HW, Yuk HJ, et al. Geranylated flavonoids displaying SARS-CoV papain-like protease inhibition from the fruits of *Paulownia tomentosa*. *Bioorganic Med Chem.* (2013) 21:3051–7. doi: 10.1016/j.bmc.2013.03.027
124. Lv J, Yu S, Wang Y, Wang D, Zhu H, Cheng R, et al. Anti-hepatitis B virus norbisabolane sesquiterpenoids from *Phyllanthus acidus* and the establishment of their absolute configurations using theoretical calculations. *J Org Chem.* (2014) 79:5432–47. doi: 10.1021/jo5004604
125. Ravikumar YS, Ray U, Nandhitha M, Perween A, Raja Naika H, Khanna N, et al. Inhibition of hepatitis C virus replication by herbal extract: *Phyllanthus amarus* as potent natural source. *Virus Res.* (2011) 158:89–97. doi: 10.1016/j.virusres.2011.03.014
126. Tan WC, Jaganath IB, Manikam R, Sekaran SD. Evaluation of antiviral activities of four local Malaysian *phyllanthus* species against herpes simplex viruses and possible antiviral target. *Int J Med Sci.* (2013) 10:1817–29. doi: 10.7150/ijms.6902
127. Zhang X, Yang LM, Liu GM, Liu YJ, Zheng CB, Lv YJ, et al. Potent anti-HIV activities and mechanisms of action of a pine cone extract from *Pinus yunnanensis*. *Molecules.* (2012) 17:6916–29. doi: 10.3390/molecules17066916
128. Hsu WC, Chang SP, Lin LC, Li CL, Richardson CD, Lin CC, et al. *Limonium sinense* and gallic acid suppress hepatitis C virus infection by blocking early viral entry. *Antiviral Res.* (2015) 118:139–47. doi: 10.1016/j.antiviral.2015.04.003
129. Chavan RD, Shinde P, Girkar K, Madage R, Chowdhary A. Assessment of Anti-Influenza activity and hemagglutination inhibition of *Plumbago*

indica and *Allium sativum* extracts. *Pharmacognosy Res.* (2016) 8:105–11. doi: 10.4103/0974-8490.172562
130. Xiong HR, Luo J, Hou W, Xiao H, Yang ZQ. The effect of Emodin, an anthraquinone derivative extracted from the roots of *Rheum tanguticum*, against herpes simplex virus *in vitro* and *in vivo*. *J Ethnopharmacol.* (2011) 133:718–23. doi: 10.1016/j.jep.2010.10.059
131. Su M, Li Y, Leung KT, Cen Y, Li T, Chen R, et al. Antiviral activity and constituent of Ardisia Chinensis Benth against coxsackie B3 virus. *Phytother Res.* (2006) 20:634–9. doi: 10.1002/ptr.1912
132. Hossan MS, Fatima A, Rahmatullah M, Khoo TJ, Nissapatorn V, Galochkina AV, et al. Antiviral activity of *Embelia ribes* Burm. f. against influenza virus *in vitro*. *Arch Virol.* (2018) 163:2121–31. doi: 10.1007/s00705-018-3842-6
133. Hung TC, Jassey A, Lin CJ, Liu CH, Lin CC, Yen MH, et al. Methanolic extract of *rhizoma coptidis* inhibits the early viral entry steps of hepatitis C virus infection. *Viruses.* (2018) 10:669. doi: 10.3390/v10120669
134. Kim HB, Lee CY, Kim SJ, Han JH, Choi KH. Medicinal herb extracts ameliorate impaired growth performance and intestinal lesion of newborn piglets challenged with the virulent porcine epidemic diarrhea virus. *J Anim Sci Technol.* (2015) 57:33. doi: 10.1186/s40781-015-0065-1
135. *Shin* WJ, Lee KH, Park MH, Seong BL. Broad-spectrum antiviral effect of *Agrimonia pilosa* extract on influenza viruses. *Microbiol Immunol.* (2010) 54:11–9. doi: 10.1111/j.1348-0421.2009.00173.x
136. Bisignano C, Mandalari G, Smeriglio A, Trombetta D, Pizzo MM, Pennisi R, et al. Almond skin extracts abrogate HSV-1 replication by blocking virus binding to the cell. *Viruses.* (2017) 9:178. doi: 10.3390/v9070178
137. Ratnoglik SL, Aoki C, Sudarmono P, Komoto M, Deng L, Shoji I, et al. Antiviral activity of extracts from *Morinda citrifolia* leaves and chlorophyll catabolites, pheophorbide a and pyropheophorbide a, against hepatitis C virus. *Microbiol Immunol.* (2014) 58:188–94. doi: 10.1111/1348-0421.12133
138. Pratheeba T, Taranath V, Sai Gopal DVR, Natarajan D. Antidengue potential of leaf extracts of *Pavetta tomentosa* and *Tarenna asiatica* (Rubiaceae) against dengue virus and its vector *Aedes aegypti* (Diptera: Culicidae). *Heliyon.* (2019) 5:e02732. doi: 10.1016/j.heliyon.2019.e02732
139. Brijesh S, Daswani P, Tetali P, Antia N, Birdi T. Studies on the antidiarrhoeal activity of *Aegle marmelos* unripe fruit : validating its traditional usage. *BMC Complement Altern Med.* (2009) 12:47. doi: 10.1186/1472-6882-9-47
140. Apriyanto DR, Aoki C, Hartati S, Hanafi M, Kardono LBS, Arsianti A, et al. Anti-hepatitis C virus activity of a crude extract from longan (*Dimocarpus longan Lour.*) leaves. *Jpn J Infect Dis.* (2016) 69:213–20. doi: 10.7883/yoken.JJID.2015.107
141. Song JH, Ahn JH, Kim SR, Cho S, Hong EH, Kwon BE, et al. Manassantin B shows antiviral activity against coxsackievirus B3 infection by activation of the STING/TBK-1/IRF3 signalling pathway. *Sci Rep.* (2019) 9:9413. doi: 10.1038/s41598-019-45868-8
142. Wang GW, Hu WT, Huang BK, Qin LP. Illicium verum: a review on its botany, traditional use, chemistry and pharmacology. *J Ethnopharmacol.* (2011) 136:10–20. doi: 10.1016/j.jep.2011.04.051
143. Abdelgawad AAM. *Tamarix nilotica* (Ehrenb) bunge: a review of phytochemistry and pharmacology. *J Microb Biochem Technol.* (2017) 9:544–53. doi: 10.4172/1948-5948.1000340
144. Ryu YB, Jeong HJ, Kim JH, Kim YM, Park JY, Kim D, et al. Biflavonoids from *Torreya nucifera* displaying SARS-CoV $3CL^{pro}$ inhibition. *Bioorganic Med Chem.* (2010) 18:7940–7. doi: 10.1016/j.bmc.2010.09.035
145. Karamese M, Aydogdu S, Karamese SA, Altoparlak U. Preventive effects of a major component of green tea, Epigallocathechin-3-Gallate, on Hepatitis-B virus DNA replication. *Asian Pac J Cancer Prev.* (2015) 16:40199–202. doi: 10.7314/APJCP.2015.16.10.4199
146. Xu J, Wang J, Deng F, Hu Z, Wang H. Green tea extract and its major component epigallocatechin gallate inhibits hepatitis B virus *in vitro*. *Antiviral Res.* (2008) 78:242–9. doi: 10.1016/j.antiviral.2007.11.011
147. Dai J, Tao H, Min Q, Zhu Q. Anti-hepatitis B virus activities of friedelolactones from *Viola diffusa Ging*. *Phytomedicine.* (2015) 22:724–9. doi: 10.1016/j.phymed.2015.05.001
148. Kwon HJ, Kim HH, Yoon SY, Ryu YB, Chang JS, Cho KO, et al. *In vitro* inhibitory activity of *Alpinia katsumadai* extracts against influenza virus infection and hemagglutination. *Virol J.* (2010) 7:307. doi: 10.1186/1743-422X-7-307
149. Thuy BTP, My TTA, Hai NTT, Hieu LT, Hoa TT, Thi Phuong Loan H, et al. Investigation into SARS-CoV-2 resistance of compounds in garlic essential oil. *ACS Omega.* (2020) 5:8312–8320. doi: 10.1021/acsomega.0c00772
150. Kim DW, Seo KH, Curtis-Long MJ, Oh KY, Oh JW, Cho JK, et al. Phenolic phytochemical displaying SARS-CoV papain-like protease inhibition from the seeds of *Psoralea corylifolia*. *J Enzyme Inhib Med Chem.* (2014) 29:59–63. doi: 10.3109/14756366.2012.753591
151. Li SY, Chen C, Zhang HQ, Guo HY, Wang H, Wang L, et al. Identification of natural compounds with antiviral activities against SARS-associated coronavirus. *Antiviral Res.* (2005) 67:18–23. doi: 10.1016/j.antiviral.2005.02.007
152. Ulasli M, Gurses SA, Bayraktar R, Yumrutas O, Oztuzcu S, Igci M, et al. The effects of *Nigella sativa* (Ns), *Anthemis hyalina* (Ah) *and Citrus sinensis* (Cs) extracts on the replication of coronavirus and the expression of TRP genes family. *Mol Biol Rep.* (2014) 41:1703–11. doi: 10.1007/s11033-014-3019-7
153. Ryu YB, Park SJ, Kim YM, Lee JY, Seo WD, Chang JS, et al. SARS-CoV $3CL^{pro}$ inhibitory effects of quinone-methide triterpenes from *Tripterygium regelii*. *Bioorganic Med Chem Lett.* (2010) 20:1873–76. doi: 10.1016/j.bmcl.2010.01.152
154. Loizzo MR, Saab AM, Tundis R, Statti GA, Menichimi F, Lampronti D, et al. Phytochemical analysis and *in vitro* antiviral activities of the essential oils of seven Lebanon species. *Chem Biodivers.* (2008) 5:461–70. doi: 10.1002/cbdv.200890045
155. Wu C, Liu Y, Yang Y, Zhang P, Zhong W, Wang Y, et al. Analysis of therapeutic targets for SARS-CoV-2 and discovery of potential drugs by computational methods. *Acta Pharm Sin B.* (2020) 10:766–88. doi: 10.1016/j.apsb.2020.02.008
156. Wen CC, Shyur LF, Jan JT, Liang PH, Kuo CJ, Arulselvan P, et al. Traditional Chinese medicine herbal extracts of *Cibotium barometz*, *Gentiana scabra*, *Dioscorea batatas*, *Cassia tora*, and *Taxillus chinensis* inhibit SARS-CoV replication. *J Tradit Complement Med.* (2011) 1:41–50. doi: 10.1016/S2225-4110(16)30055-4
157. Zhuang M, Jiang H, Suzuki Y, Li X, Xiao P, Tanaka T, et al. Procyanidins and butanol extract of Cinnamomi Cortex inhibit SARS-CoV infection. *Antiviral Res.* (2009) 82:73–81. doi: 10.1016/j.antiviral.2009.02.001
158. Ho TY, Wu SL, Chen JC, Li CC, Hsiang CY. Emodin blocks the SARS coronavirus spike protein and angiotensin-converting enzyme 2 interaction. *Antiviral Res.* (2007) 74:92–101. doi: 10.1016/j.antiviral.2006.04.014
159. Luo W, Su X, Gong S, Qin Y, Liu W, Li J, et al. Anti-SARS coronavirus 3C-like protease effects of *Rheum palmatum* L. extracts. *Biosci Trends.* (2009) 3:124–6.
160. Yang C, Lee Y, Hsu H, Shih C, Chao Y, Lee S. Targeting coronaviral replication and cellular JAK2 mediated dominant NF- κB activation for comprehensive and ultimate inhibition of coronaviral activity. *Sci Rep.* (2017) 7:4105. doi: 10.1038/s41598-017-04203-9
161. Adhikari SP, Meng S, Wu Y, Mao Y, Ye R, Wang Q, et al. Novel Coronavirus during the early outbreak period: epidemiology, causes, clinical manifestation and diagnosis, prevention and control. *Infect Dis Poverty.* (2020) 9:29. doi: 10.1186/s40249-020-00646-x
162. Nickbakhsh S, Ho A, Marques DFP, McMenamin J, Gunson RN, Murcia PR. Epidemiology of seasonal coronaviruses: establishing the context for the emergence of Coronavirus disease 2019. *J Infect Dis.* (2020) 222:17–25. doi: 10.1093/infdis/jiaa185
163. Wu D, Wu T, Liu Q, Yang Z. The SARS-CoV-2 outbreak: what we know. *Int J Infect Dis.* (2020) 94:44–8. doi: 10.1016/j.ijid.2020.03.004
164. Li Q, Guan X, Wu P, Wang X, Zhou L, Tong Y, et al. Early transmission dynamics in Wuhan, China, of novel coronavirus-infected pneumonia. *N Engl J Med.* (2020) 382:1199–207. doi: 10.1056/NEJMoa2001316
165. Phan LT, Nguyen TV, Luong QC, Nguyen TV, Nguyen TV, Nguyen HT, et al. Importation and human-to-human transmission of a novel Coronavirus in Vietnam. *N Engl J Med.* (2020) 382:872–4. doi: 10.1056/NEJMc2001272
166. Cots JM, Alós J, Bárcena M, Boleda X. COVID-19 in Brazil: "So what?". *Lancet.* (2020) 395:1461. doi: 10.1016/S0140-6736(20)31095-3
167. *COVID-19 Dashboard by the Center for Systems Science and Engineering (CSSE) at Johns Hopkins University (JHU).* Available online at: https://coronavirus.jhu.edu/map.html (accessed July 5, 2020).

168. Yang H, Bartlam M, Rao Z. Drug design targeting the main protease, the Achilles Heel of coronaviruses. *Curr Pharm Des.* (2006) 12:4573–90. doi: 10.2174/138161206779010369
169. Mousavizadeh L, Ghasemi S. Genotype and phenotype of COVID-19: their roles in pathogenesis. *J Microbiol Immunol Infect.* (2020). doi: 10.1016/j.jmii.2020.03.022. [Epub ahead of print].
170. Ou X, Liu Y, Lei X, Li P, Mi D, Ren L, et al. Characterization of spike glycoprotein of SARS-CoV-2 on virus entry and its immune cross-reactivity with SARS-CoV. *Nat Commun.* (2020) 11:1620. doi: 10.1038/s41467-020-15562-9
171. Coutard B, Valle C, de Lamballerie X, Canard B, Seidah NG, Decroly E. The spike glycoprotein of the new coronavirus 2019-nCoV contains a furin-like cleavage site absent in CoV of the same clade. *Antiviral Res.* (2020) 176:140742. doi: 10.1016/j.antiviral.2020.104742
172. Liang Y, Wang M, Chien C, Yarmishyn AA. Highlight of immune pathogenic response and hematopathologic effect in SARS-CoV, MERS-CoV and SARS-Cov-2 infection. *Front Immunol.* (2020) 11:1022. doi: 10.3389/fimmu.2020.01022
173. Chen B, Tian E, He B, Tian L, Han R, Wang S, et al. Overview of lethal human coronaviruses. *Sig Transduct Target Ther.* (2020) 5:89. doi: 10.1038/s41392-020-0190-2
174. Zhou Y, Hou Y, Shen J, Huang Y, Martin W, Cheng F. Network-based drug repurposing for novel coronavirus 2019-nCoV/SARS-CoV-2. *Cell Discov.* (2020) 6:14. doi: 10.1038/s41421-020-0153-3
175. Xu J, Zhao S, Teng T, Abdalla AE, Zhu W, Xie L, et al. Systematic comparison of two animal-to-human transmitted human Coronaviruses: SARS-CoV-2 and SARS-CoV. *Viruses.* (2020) 12:244. doi: 10.3390/v12020244
176. Prajapat M, Sarma P, Shekhar N, Avti P, Sinha S, Kaur H, et al. Drug targets for corona virus: a systematic review. *Indian J Pharmacol.* (2020) 52:56–65. doi: 10.4103/ijp.IJP_115_20
177. Rabaan AA, Al-Ahmed SH, Haque S, Sah R, Tiwari R, Malik YS, et al. SARS-CoV-2, SARS-CoV, and MERS-COV: a comparative overview. *Le Infez Med.* (2020) 28:174–84.
178. Tang X, Wu C, Li X, Song Y, Yao X, Wu X, et al. On the origin and continuing evolution of SARS-CoV-2. *Natl Sci Rev.* (2020) 3:nwaa036. doi: 10.1093/nsr/nwaa036
179. Zhang H, Penninger JM, Li Y, Zhong N, Slutsky AS. Angiotensin-converting enzyme 2 (ACE2) as a SARS-CoV-2 receptor: molecular mechanisms and potential therapeutic target. *Intensive Care Med.* (2020) 46:586–90. doi: 10.1007/s00134-020-05985-9
180. Hoffmann M, Kleine-Weber H, Schroeder S, Krüger N, Herrler T, Erichsen S, et al. SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor. *Cell.* (2020) 2:52. doi: 10.1016/j.cell.2020.02.052
181. Skariayachan S, Challapilli SB, Packkirisamy S, Kumargowda ST, Sridhar VS. Recent aspects on the pathogenesis mechanism, animal models and novel therapeutics interventions for Middle East respiratory syndrome coronavirus infections. *Front Microbiol.* (2019) 10:569. doi: 10.3389/fmicb.2019.00569
182. Shereen MA, Khan S, Kazmi A, Bashir N, Siddique R. COVID-19 infection: origin, transmission, and characteristics of human coronaviruses. *J Adv Res.* (2020) 24:91–8. doi: 10.1016/j.jare.2020.03.005
183. Rampersad S, Tennant P. Replication and expression strategies of viruses. *Viruses.* (2018) 2018:55–82. doi: 10.1016/B978-0-12-811257-1.00003-6
184. Wang H, Xue S, Yang H, Chen C. Recent progress in the discovery of inhibitors targeting coronavirus proteases. *Virol Sin.* (2016) 31:24–30. doi: 10.1007/s12250-015-3711-3
185. Cao W, Li T. COVID-19: towards understanding of pathogenesis. *Cell Res.* (2020) 30:367–9. doi: 10.1038/s41422-020-0327-4
186. Rockx B, Kuiken T, Herfst S, Bestebroer T, Lamers MM, Oude Munnink BB, et al. Comparative pathogenesis of COVID-19, MERS, and SARS in a nonhuman primate model. *Science.* (2020) 368:1012–5. doi: 10.1126/science.abb7314
187. Chen Y, Liu Q, Guo D. Emerging coronaviruses: genome structure, replication, and pathogenesis. *J Med Virol.* (2020) 92:418–23. doi: 10.1002/jmv.25681
188. Kim D, Lee JY, Yang JS, Kim JW, Kim VN, Chang H. The architecture of SARS-CoV-2 transcriptome. *Cell.* (2020) 181:914–21. doi: 10.1016/j.cell.2020.04.011
189. Shi Y, Wang Y, Shao C, Huang J, Gan J, Huang X, et al. COVID-19 infection: the perspectives on immune responses. *Cell Death Differ.* (2020) 27:1451–4. doi: 10.1038/s41418-020-0530-3
190. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet.* (2020) 395:497–506. doi: 10.1016/S0140-6736(20)30183-5
191. Rothan HA, Byrareddy SN. The epidemiology and pathogenesis of coronavirus disease (COVID-19) outbreak. *J Autoimmun.* (2020) 109:102433. doi: 10.1016/j.jaut.2020.102433
192. Devaux CA, Rolain JM, Raoult D. ACE2 receptor polymorphism: susceptibility to SARS-CoV-2, hypertension, multi-organ failure, and COVID-19 disease outcome. *J Microbiol Immunol Infect.* (2020) 53:425–35. doi: 10.1016/j.jmii.2020.04.015
193. Tahir ul Qamar M, Alqahtani SM, Alamri MA, Chen LL. Structural basis of SARS-CoV-2 3CLpro and anti-COVID-19 drug discovery from medicinal plants. *J Pharm Anal.* (in press) doi: 10.20944/preprints202002.0193.v1
194. Shang J, Ye G, Shi K, Wan Y, Luo C, Aihara H, et al. Structural basis of receptor recognition by SARS-CoV-2. *Nature.* (2020) 581:221–4. doi: 10.1038/s41586-020-2179-y
195. Hilgenfeld R. From SARS to MERS: crystallographic studies on coronaviral proteases enable antiviral drug design. *FEBS J.* (2014) 281:4085–96. doi: 10.1111/febs.12936
196. Zeng Q, Langereis MA, van Vliet ALW, Huizinga EG, de Groot RJ. Structure of coronavirus hemagglutinin-esterase offers insight into corona and influenza virus evolution. *Proc Natl Acad Sci USA.* (2008) 105:9065–9. doi: 10.1073/pnas.0800502105
197. Wang W, Xu Y, Gao R, Lu R, Han K, Wu G, et al. Detection of SARS-CoV-2 in different types of clinical specimens. *JAMA.* (2020) 1843–44. doi: 10.1001/jama.2020.3786
198. Saxena SK, Kumar S, Maurya VK, Sharma R, Dandu HR, Bhatt MLB. Current insight into the novel coronavirus disease 2019 (COVID-19). *Nat Public Health Emerg Collect.* (2020) 2019:1–8. doi: 10.1007/978-981-15-4814-7_1
199. Liu C, Zhou Q, Li Y, Garner LV, Watkins SP, Carter LJ, et al. Research and development on therapeutic agents and vaccines for covid-19 and related human coronavirus diseases. *ACS Cent Sci.* (2020) 6:315–31. doi: 10.1021/acscentsci.0c00272
200. Salata C, Calistri A, Parolin C, Palù G. Coronaviruses: a paradigm of new emerging zoonotic diseases. *Pathog Dis.* (2020) 77:ftaa006. doi: 10.1093/femspd/ftaa006
201. Wilder-smith A, Chiew CJ, Lee VJ. Can we contain the COVID-19 outbreak with the same measures as for SARS? *Lancet Infect Dis.* (2020) 20:e102–7. doi: 10.1016/S1473-3099(20)30129-8
202. Li G, De Clercq E. Therapeutic options for the 2019 novel coronavirus (2019-nCoV). *Nat Rev Drug Discov.* (2020) 19:149–50. doi: 10.1038/d41573-020-00016-0
203. Andersen PI, Ianevski A, Lysvand H, Vitkauskiene A, Oksenych V, Bjørås M, et al. Discovery and development of safe-in-man broad-spectrum antiviral agents. *Int J Infect Dis.* (2020) 93:268–76. doi: 10.1016/j.ijid.2020.02.018
204. Liu M, Yu Q, Xiao H, Yi Y, Cheng H, Putra DF, et al. Antiviral activity of *Illicium verum Hook*. f. extracts against grouper iridovirus infection. *J Fish Dis.* (2020) 43:531–40. doi: 10.1111/jfd.13146
205. Fuzimoto AD, Isidoro C. The antiviral and the coronavirus-host protein pathways inhibiting properties of herbs and natural compounds - additional weapons in the fight against the COVID-19 pandemic? *J Tradit Complement Med.* (2020) 10:405–19. doi: 10.1016/j.jtcme.2020.05.003
206. Chen L, Xiong J, Bao L, Shi Y. Convalescent plasma as a potential therapy for COVID-19. *Lancet Infect Dis.* (2020) 20:398–400. doi: 10.1016/S1473-3099(20)30141-9
207. Cao X. COVID-19: immunopathology and its implications for therapy. *Nat Rev Immunol.* (2020) 20:269–70. doi: 10.1038/s41577-020-0308-3
208. Peng S, Li Z, Zou L, Liu W, Liu C, McClements DJ. Enhancement of curcumin bioavailability by encapsulation in Sophorolipid-coated nanoparticles: an *in vitro* and *in vivo* study. *Agric Food Chem.* (2018) 66:1488–97. doi: 10.1021/acs.jafc.7b05478
209. Vankadari N. Arbidol: a potential antiviral drug for the treatment of SARS-CoV-2 by blocking trimerization of the spike glycoprotein. *Int J Antimicrob Agents.* (2020) 28:105998. doi: 10.1016/j.ijantimicag.2020.105998

210. Khan SA, Zia K, Ashraf S, Uddin R, Ul-Haq Z. Identification of chymotrypsin-like protease inhibitors of SARS-CoV-2 via integrated computational approach. *J Biomol Struct Dyn.* (2020) 2020:1–10. doi: 10.1080/07391102.2020.1751298
211. Delang L, Abdelnabi R, Neyts J. Favipiravir as a potential countermeasure against neglected and emerging RNA viruses. *Antiviral Res.* (2018) 153:85–94. doi: 10.1016/j.antiviral.2018.03.003
212. Wang M, Cao R, Zhang L, Yang X, Liu J, Xu M. Remdesivir and chloroquine effectively inhibit the recently emerged novel coronavirus (2019-nCoV) *in vitro*. *Cell Res.* (2020) 30:269–71. doi: 10.1038/s41422-020-0282-0
213. Caly L, Druce JD, Catton MG, Jans DA, Wagstaff KM. The FDA-approved drug ivermectin inhibits the replication of SARS-CoV-2 *in vitro*. *Antiviral Res.* (2020) 178:3–6. doi: 10.1016/j.antiviral.2020.104787
214. Cao B, Wang Y, Wen D, Liu W, Wang J, Fan G, et al. A trial of lopinavir-ritonavir in adults hospitalized with severe covid-19. *N Engl J Med.* (2020) 382:1787–99. doi: 10.1056/NEJMc2008043
215. Elmezayen AD, Al-Obaidi A, Sahin AT, Yelekçi K. Drug repurposing for coronavirus (COVID-19): *in silico* screening of known drugs against coronavirus 3CL hydrolase and protease enzymes. *J Biomol Struct Dyn.* (2020) 2020:1–13. doi: 10.1080/07391102.2020.1758791
216. Muralidharan N, Sakthivel R, Velmurugan D, Gromiha MM. Computational studies of drug repurposing and synergism of lopinavir, oseltamivir and ritonavir binding with SARS-CoV-2 protease against COVID-19. *J Biomol Struct Dyn*. (2020) 2020:1–6. doi: 10.1080/07391102.2020.1752802
217. Dong L, Hu S, Gao J. Discovering drugs to treat coronavirus disease 2019 (COVID-19). *Drug Discov Ther*. (2020) 14:58–60. doi: 10.5582/ddt.2020.01012
218. Sarma P, Sekhar N, Prajapat M, Avti P, Kaur H, Kumar S, et al. *In-silico* homology assisted identification of inhibitors of RNA binding against 2019-nCoV N-protein (N terminal domain). *J Biomol Struct Dyn.* (2020) 2020:1–9. doi: 10.1080/07391102.2020.1753580
219. Baron SA, Devaux C, Colson P, Raoult D, Rolain JM. Teicoplanin: an alternative drug for the treatment of COVID-19? *Int J Antimicrob Agents.* (2020) 55:105944. doi: 10.1016/j.ijantimicag.2020.105944
220. Enmozhi SK, Raja K, Sebastine I, Joseph J. Andrographolide as a potential inhibitor of SARS-CoV-2 main protease: an *in silico* approach. *J Biomol Struct Dyn.* (2020) 2020:1–7. doi: 10.1080/07391102.2020.1760136
221. Wu R, Wang L, Kuo HCD, Shannar A, Peter R, Chou PJ, et al. An update on current therapeutic drugs treating COVID-19. *Curr Pharmacol Rep.* (2020) 11:1–15. doi: 10.1007/s40495-020-00216-7
222. Maurya VK, Kumar S, Bhatt MLB, Saxena SK. Therapeutic development and drugs for the treatment of COVID-19. *Nat Public Health Emerg Collect.* (2020) 2019:109–26. doi: 10.1007/978-981-15-4814-7_10
223. Koparde AA, Doijad RC, Magdum CS, Koparde AA. Natural products in drug discovery. In: Doijad RC, editor. *Pharmacognosy - Medicinal Plants*. Rijeka: IntechOpen. (2019). p. 14. doi: 10.5772/intechopen.82860
224. Ekor M. The growing use of herbal medicines: issues relating to adverse reactions and challenges in monitoring safety. *Front Neurol.* (2014) 4:177. doi: 10.3389/fphar.2013.00177
225. Ganjhu RK, Mudga PP, Maity H, Dowarha D, Devadiga S, Nag S, et al. Herbal plants and plant preparations as a remedial approach for viral diseases. *Virus Dis.* (2015) 26:225–36. doi: 10.1007/s13337-015-0276-6
226. Chitemerere TA, Mukanganyama S. Evaluation of cell membrane integrity as a potential antimicrobial target for plant products. *BMC Complement Altern Med.* (2014) 14:278. doi: 10.1186/1472-6882-14-278
227. Anandhi D, Srinivasan PT, Kumar GP, Jagatheesh S. Original research article DNA fragmentation induced by the glycosides and flavonoids from *C. coriaria*. *Int J Curr Microbiol App Sci.* (2014) 3:666–73.
228. Zhao X, Zhao F, Zhong N. Quorum sensing inhibition and anti-biofilm activity of traditional Chinese medicine. In: El-Samragy Y, editor. *Food Safety - Some Global Trends*. Itratech Open (2018). p. 14. doi: 10.5772/intechopen.74658
229. Radulovic NS, Blagojevic PD, Stojanovic-Radic ZZ, Stojanovic NM. Antimicrobial plant metabolites: structural diversity and mechanism of action. *Curr Med Chem.* (2013) 20:932–52. doi: 10.2174/0929867311320070008
230. Mogosanu GD, Grumezescu AM, Huang KS, Bejenaru LE, Bejenaru C. Prevention of microbial communities: novel approaches based natural products. *Curr Pharm Biotechnol.* (2015) 16:94–111. doi: 10.2174/1389201016021501129 16
231. Anand U, Jacobo-Herrera N, Altemimi A, Lakhssassi N. A comprehensive review on medicinal plants as antimicrobial therapeutics: potential avenues of biocompatible drug discovery. *Metabolites.* (2019) 9:258. doi: 10.3390/metabo9110258
232. Zhang D, Wu K, Zhang X, Deng S, Peng B. *In silico* screening of Chinese herbal medicines with the potential to directly inhibit 2019 novel coronavirus. *J Integr Med.* (2020) 18:152–8. doi: 10.1016/j.joim.2020.02.005
233. Kiran G, Karthik L, Shree Devi MS, Sathiyarajeswaran P, Kanakavalli K, Kumar KM, et al. *In silico* computational screening of Kabasura Kudineer - official Siddha Formulation and JACOM against SARS-CoV-2 spike protein. *J Ayurveda Integr Med*. (in press) doi: 10.1016/j.jaim.2020.05.009
234. Subbaiyan A, Ravichandran K, Singh SV, Sankar M, Thomas P, Dhama K, et al. *In silico* molecular docking analysis targeting SARS-CoV-2 spike protein and selected herbal constituents. *J Pure Appl Microbiol.* (2020) 14:989–98. doi: 10.22207/JPAM.14.SPL1.37
235. Nivetha R, Bhuvaragavan S, Janarthanan S. Inhibition of multiple SARS-CoV-2 proteins by an antiviral biomolecule, seselin from *Aegle marmelos* deciphered using molecular docking analysis. *Res Sq*. (2020) 2020:v1. doi: 10.21203/rs.3.rs-31134/v1
236. Basu A, Sarkar A, Maulik U. Computational approach for the design of potential spike protein binding natural compounds in SARS- CoV2. *Pharmacodynamics*. (2020). doi: 10.21203/rs.3.rs-33181/v1. [Epub ahead of print].
237. Krishnasamy R, Anand T, Baba M, Bharath MV, Phuntsho J, Arunachalam D, et al. *In silico* analysis of active compounds from siddha herbal infusion of Ammaiyar Koondhal Kudineer (Akk) against SARS-CoV-2 spike protein and its ACE2 receptor complex. *SSRN Online J.* (2020). doi: 10.2139/ssrn.3578294. [Epub ahead of print].
238. Pandit M, Latha N. *In silico* studies reveal potential antiviral activity of phytochemicals from medicinal plants for the treatment of COVID-19 infection. *Res Sq*. (2020). doi: 10.21203/rs.3.rs-22687/v1. [Epub ahead of print].
239. Pandeya KB, Ganeshpurkar A, Kumar Mishra M. Natural RNA dependent RNA polymerase inhibitors: molecular docking studies of some biologically active alkaloids of *Argemone mexicana*. *Med Hypotheses.* (2020) 2020:109905. doi: 10.1016/j.mehy.2020.109905
240. Aanouz I, Belhassan A, El-Khatabi K, Lakhlifi T, El-ldrissi M, Bouachrine M. Moroccan medicinal plants as inhibitors against SARS-CoV-2 main protease: computational investigations. *J Biomol Struct Dyn*. (2020) 2020:1–9. doi: 10.1080/07391102.2020.1758790
241. Bhardwaj VK, Singh R, Sharma J, Rajendran V, Purohit R, Kumar S. Identification of bioactive molecules from tea plant as SARS-CoV-2 main protease inhibitors. *J Biomol Struct Dyn*. (2020) 2020:1–11. doi: 10.1080/07391102.2020.1766572
242. Serseg T, Benarous K, Yousfi M. Hispidin and Lepidine E: two natural compounds and folic acid as potential inhibitors of 2019-novel coronavirus main protease (2019-nCoVMpro), molecular docking and SAR study. *Curr Comput Aided Drug Des.* (2020). doi: 10.2174/1573409916666200422075440. [Epub ahead of print].
243. Kumar A, Choudhir G, Shukla SK, Sharma M, Tyagi P, Bhushan A, et al. Identification of phytochemical inhibitors against main protease of COVID-19 using molecular modeling approaches. *J Biomol Struct Dyn*. (2020) 2020:1–11. doi: 10.21203/rs.3.rs-31210/v1
244. Islam MT, Sarkar C, El-Kersh DM, Jamaddar S, Uddin SJ, Shilpi JA, et al. Natural products and their derivatives against coronavirus: a review of the non-clinical and pre-clinical data. *Phyther Res*. (2020). doi: 10.1002/ptr.6700. [Epub ahead of print].
245. Sayed AM, Khattab AR, AboulMagd AM, Hassan HM, Rateb ME, Zaid H, et al. Nature as a treasure trove of potential anti-SARS-CoV drug leads: a structural/mechanistic rationale. *RSC Adv.* (2020) 10:19790–802. doi: 10.1039/D0RA04199H
246. Sepay N, Sepay N, Al Hoque A, Mondal R, Halder UC, Muddassir M. *In silico* fight against novel coronavirus by finding chromone derivatives as inhibitor of coronavirus main proteases enzyme. *Struct Chem.* (2020) 2020:1–10. doi: 10.1007/s11224-020-01537-5

247. Umesh U, Kundu D, Selvaraj C, Singh SK, Dubey VK. Identification of new anti-nCoV drug chemical compounds from Indian spices exploiting SARS-CoV-2 main protease as target. *J Biomol Struct Dyn.* (2020) 2020:1–9. doi: 10.1080/07391102.2020.1763202
248. Singh A, Mishra A. Leucoefdin a potential inhibitor against SARS CoV-2 M. *J Biomol Struct Dyn*. (2020) 2020:1–6. doi: 10.1080/07391102.2020.1777903
249. Gupta MK, Vemula S, Donde R, Gouda G, Behera L, Vadde R. *In-silico* approaches to detect inhibitors of the human severe acute respiratory syndrome coronavirus envelope protein ion channel. *J Biomol Struct Dyn*. (2020) 2020:1–11. doi: 10.1080/07391102.2020.1751300
250. Joshi RS, Jagdale SS, Bansode SB, Shankar SS, Tellis MB, Pandya VK, et al. Discovery of potential multi-target-directed ligands by targeting host-specific SARS-CoV-2 structurally conserved main protease. *J Biomol Struct Dyn*. (2020) 2020:1–6. doi: 10.20944/preprints202004.0068.v2
251. Joshi T, Joshi T, Sharma P, Mathpal S, Pundir H, Bhatt V, et al. *In silico* screening of natural compounds against COVID-19 by targeting Mpro and ACE2 using molecular docking. *Eur Rev Med Pharmacol Sci.* (2020) 24:4529–36. doi: 10.26355/eurrev_202004_21036
252. Salman S, Shah FH, Idrees J, Idrees F, Velagala S, Ali J, et al. Virtual screening of immunomodulatory medicinal compounds as promising anti-SARS-COV-2 inhibitors. *Future Virol.* (2020). doi: 10.2217/fvl-2020-0079. [Epub ahead of print].
253. Wahedi HM, Ahmad S, Abbasi SW. Stilbene-based natural compounds as promising drug candidates against COVID-19. *J Biomol Struct Dyn*. (2020) 2020:1–10. doi: 10.1080/07391102.2020.1762743
254. Abdelli I, Hassani F, Bekkel Brikci S, Ghalem S. *In silico* study the inhibition of angiotensin converting enzyme 2 receptor of COVID-19 by *Ammoides verticillata* components harvested from Western Algeria. *J Biomol Struct Dyn*. (2020) 2020:1–14. doi: 10.1080/07391102.2020.1763199
255. Murugan NA, Pandian CJ, Jeyakanthan J. Computational investigation on *Andrographis paniculata* phytochemicals to evaluate their potency against SARS-CoV-2 in comparison to known antiviral compounds in drug trials. *J Biomol Struct Dyn*. (2020) 2020:1–12. doi: 10.1080/07391102.2020.1777901
256. Gyebi GA, Ogunro OB, Adegunloye AP, Ogunyemi OM, Afolabi SO. Potential inhibitors of coronavirus 3-chymotrypsin-like protease ($3CL^{pro}$): an *in silico* screening of alkaloids and Terpenoids from African medicinal plants. *J Biomol Struct Dyn.* (2020) 20201–13. doi: 10.1080/07391102.2020.1764868
257. Sampangi-Ramaiah MH, Vishwakarma R, Shaanker RU. Molecular docking analysis of selected natural products from plants for inhibition of SARS-CoV-2 main protease. *Curr Sci.* (2020) 118:1087–92.
258. Kumar V, Dhanjal JK, Bhargava P, Kaul A, Wang J, Zhang H, et al. Withanone and withaferin-A are predicted to interact with transmembrane protease serine 2 (TMPRSS2) and block entry of SARS-CoV-2 into cells. *J Biomol Struct Dyn*. (2020) 2020:1–13. doi: 10.1080/07391102.2020.1775704
259. Borkotoky S, Banerjee M. A computational prediction of SARS-CoV-2 structural protein inhibitors from *Azadirachta indica* (Neem). *J Biomol Struct Dyn*. (2020) 2020:1–7. doi: 10.1080/07391102.2020.1774419
260. Elfiky AA. Natural products may interfere with SARS-CoV-2 attachment to the host cell. *J Biomol Struct Dyn*. (2020) 2020:1–10. doi: 10.21203/rs.3.rs-22458/v1
261. Ahmad S, Abbasi HW, Shahid S, Gul S, Abbasi SW. Molecular docking, simulation and MM-PBSA studies of nigella sativa compounds: a computational quest to identify potential natural antiviral for COVID-19 treatment. *J Biomol Struct Dyn*. (2020) 2020:1–9. doi: 10.1080/07391102.2020.1775129
262. Saito K. Phytochemical genomics-a new trend. *Curr Opin Plant Biol.* (2013) 16:373–80. doi: 10.1016/j.pbi.2013.04.001
263. Mathur S, Hoskins C. Drug development: lessons from nature. *Biomed Rep.* (2017) 6:612–4. doi: 10.3892/br.2017.909
264. Wink M. Plant secondary metabolites modulate insect behavior-steps toward addiction? *Front Physiol.* (2018) 9:364. doi: 10.3389/fphys.2018.00364
265. Ebenezer KS, Manivannan R, Punniyamoorthy A, Tamilselvan C. Plant secondary metabolites of antiviral properties a rich medicinal source for drug discovery: a mini review. *J Drug Deliv Ther.* (2019) 9:161–7. doi: 10.22270/jddt.v9i5.3471
266. Calixto JB. The role of natural products in modern drug discovery. *An Acad Bras Cienc.* (2019) 91:20190105. doi: 10.1590/0001-3765201920190105
267. Hardman WE. Diet components can suppress inflammation and reduce cancer risk. *Nutr Res Prac.t.* (2014) 8:233–40. doi: 10.4162/nrp.2014.8.3.233
268. Atanasov AG, Waltenberger B, Pferschy-Wenzig EM, Linder T, Wawrosch C, Uhrin P, et al. Discovery and resupply of pharmacologically active plant-derived natural products: a review. *Biotechnol Adv.* (2015) 33:1582–614. doi: 10.1016/j.biotechadv.2015.08.001
269. Annunziata G, Sanduzzi Zamparelli M, Santoro C, Ciampaglia R, Stornaiuolo M, Tenore GC, et al. May polyphenols have a role against coronavirus infection? An overview of *in vitro* evidence. *Front Med.* (2020) 7:240. doi: 10.3389/fmed.2020.00240
270. Chowdhury P, Sahuc M, Rouillé C, Rivière C, Bonneau N, Vandeputte A, et al. Theaflavins, polyphenols of black tea, inhibit entry of hepatitis C virus in cell culture. *PLoS ONE.* (2018) 13:11. doi: 10.1371/journal.pone.0198226
271. Vázquez-Calvo Á, Oya N, Martín-Acebes MA, Garcia-Moruno E, Saiz J. Antiviral properties of the natural polyphenols delphinidin and epigallocatechin gallate against the flaviviruses West Nile virus, Zika virus, and dengue virus. *Front Microbiol.* (2017) 8:1314. doi: 10.3389/fmicb.2017.01314
272. Park JY, Yuk HJ, Ryu HW, Lim SH, Kim KS, Park KH, et al. Evaluation of polyphenols from *Broussonetia papyrifera* as coronavirus protease inhibitors. *J Enzyme Inhib Med Chem.* (2017) 32:504–12. doi: 10.1080/14756366.2016.1265519
273. Abdelmageed MI, Abdelmoneim AH, Mustafa MI, Elfadol NM, Murshed NS, Shantier SW, et al. Design of a multiepitope-based peptide vaccine against the E protein of human COVID-19: an immunoinformatics approach. *BioMed Res Int.* (2020) 2020:2683286. doi: 10.1101/2020.02.04.934232
274. Ghosh R, Chakraborty A, Biswas A, Chowdhuri S. Evaluation of green tea polyphenols as novel coronavirus (SARS CoV-2) main protease (Mpro) inhibitors – an *in silico* docking and molecular dynamics simulation study. *J Biomol Struct Dyn*. (2020). doi: 10.1080/07391102.2020.1779818. [Epub ahead of print].
275. Jo S, Kim S, Shin DH, Kim MS. Inhibition of SARS-CoV 3CL protease by flavonoids. *J Enzyme Inhib Med Chem.* (2020) 35:145–51. doi: 10.1080/14756366.2019.1690480
276. Peterson L. *In silico* molecular dynamics docking of drugs to the inhibitory active site of SARS-CoV-2 protease and their predicted toxicology and ADME. *SSRN Electron J.* (2020). doi: 10.26434/chemrxiv.12155523.v1. [Epub ahead of print].
277. Owis A, Marwa EH, Dalia EA, Omar A, Usama A, Mohamed K. Molecular docking reveals the potential of *Salvadora persica* flavonoids to inhibit COVID-19 virus main protease. *RSC Adv*. (2020) 10:19570–75. doi: 10.1039/D0RA03582C
278. Thawabteh A, Juma S, Bader M, Karaman D, Scrano L, Bufo SA, et al. The biological activity of natural alkaloids against herbivores, cancerous cells and pathogens. *Toxins.* (2019) 11:656–83. doi: 10.3390/toxins11110656
279. Kim DE, Min JS, Jang MS, Lee JY, Shin YS, Park CM, et al. Natural bis-benzylisoquinoline alkaloids-tetrandrine, fangchinoline, and cepharanthine, inhibit human coronavirus oc43 infection of mrc-5 human lung cells. *Biomolecules.* (2019) 9:696. doi: 10.3390/biom9110696
280. Wink M. Potential of DNA intercalating alkaloids and other plant secondary metabolites against SARS-CoV-2 causing COVID-19. *Diversity.* (2020) 12:175. doi: 10.3390/d12050175
281. Colson P, Rolain J, Roult D. Chloroquine for the 2019 novel coronavirus SARS-CoV-2. *Int J Antimicrob Agents.* (2020) 55:105923. doi: 10.1016/j.ijantimicag.2020.105923
282. Hussein RA, El-Anssary AA. *Plants secondary metabolites: the key drivers of the pharmacological actions of medicinal plants.* Herbal Medicine. (2019). doi: 10.5772/intechopen.76139
283. Wen CC, Kuo YH, Jan JT, Liang PH, Wang SY, Liu HG, et al. Specific plant terpenoids and lignoids possess potent antiviral activities against severe acute respiratory syndrome coronavirus. *J Med Chem*. (2007) 50:4087–95. doi: 10.1021/jm070295s
284. Álvarez DM, Castillo E, Duarte LF, Arriagada J, Corrales N, Farías MA, et al. Current antivirals and novel botanical molecules interfering with herpes simplex virus infection. *Front Microbiol.* (2020) 11:139. doi: 10.3389/fmicb.2020.00139

285. Sun Z, Yu C, Wang W, Yu G, Zhang T, Zhang L, et al. Aloe polysaccharides inhibit Influenza A virus infection—a promising natural anti-flu drug. *Front Microbiol.* (2018) 9:2338. doi: 10.3389/fmicb.2018.02338
286. Mera IFG, Falconí DEG, Córdova VM. Secondary metabolites in plants: main classes, phytochemical analysis and pharmacological activities. *Rev Bionatura*. (2019) 4:1000–9. doi: 10.21931/RB/2019.04.04.11
287. Strovel J, Sittampalam S, Coussens NP, Hughes M, Inglese J, Kurtz A, et al. *Early Drug Discovery and Development Guidelines: for Academic Researchers, Collaborators, and Start-Up Companies*. Assay Guidance Manual. Bethesda, MD: Eli Lilly & Company and the National Center for Advancing Translational Sciences (2004). p. 1–35.
288. Krüger A, Gonçalves Maltarollo V, Wrenger C, Kronenberger T. ADME profiling in drug discovery and a new path paved on silica. In: Gaitonde V, editor. *Drug Discovery and Development - New Advances*. Itratech open (2020). p. 32. doi: 10.5772/intechopen.86174
289. Seca AML, Pinto DCGA. Biological potential and medical use of secondary metabolites. *Medicines.* (2019) 6:66. doi: 10.3390/medicines6020066
290. Dias DA, Urban S, Roessner U. A historical overview of natural products in drug discovery. *Metabolites.* (2012) 2:303–36. doi: 10.3390/metabo2020303
291. Anand U, Nandy S, Mundhra A, Das N, Pandey DK, Dey A. A review on antimicrobial botanicals, phytochemicals and natural resistance modifying agents from Apocynaceae family: possible therapeutic approaches against multidrug resistance in pathogenic microorganisms. *Drug Resist Update.* (2020) 51:100695. doi: 10.1016/j.drup.2020.100695
292. Anwar N, Teo, Y, Joash T. The role of plant metabolites in drug discovery: current challenges and future perspectives. In: Swamy, MK, Akhtar, MS, editors. *Natural Bio-active Compounds, Volume 2: Chemistry, Pharmacology and Health Care Practices*. New York, NY: Springer Publications (2019). p. 25–51. doi: 10.1007/978-981-13-7205-6_2
293. Gonzalez-Alfonso JL, Peñalver P, Ballesteros AO, Morales JC, Plou FJ. Effect of α-Glycosylation on the stability, antioxidant properties, toxicity, and neuroprotective activity of (-)-Epigallocatechin gallate. *Front Nutr.* (2019) 6:30. doi: 10.3389/fnut.2019.00030
294. Sharifi-Rad M, Pezzani R, Redaelli M, Zorzan M, Imran M, Khalil AA, et al. Preclinical pharmacological activities of Epigallocatechin-3-gallate in signaling pathways: an update on cancer. *Molecules.* (2020) 25:467. doi: 10.3390/molecules25030467
295. Banerjee A, Czinn SJ, Reiter RJ, Blanchard TG. Crosstalk between endoplasmic reticulum stress and anti-viral activities: a novel therapeutic target for COVID-19. *Life Sci.* (2020) 255:117842. doi: 10.1016/j.lfs.2020.117842
296. Karade PG, Jadhav NR. Colon targeted curcumin microspheres laden with ascorbic acid for bioavailability enhancement. *J Microencapsul.* (2018) 35:372–80. doi: 10.1080/02652048.2018.1501111
297. Azim KF, Ahmed SR, Banik A, Khan MMR, Deb A, Somana SR. Screening and druggability analysis of some plant metabolites against SARS-CoV-2: an integrative computational approach. *Inform Med Unlocked.* (2020) 20:100367. doi: 10.1016/j.imu.2020.100367
298. Capell T, Twyman RM, Armario NV, Ma JKC, Schillberg S, Christou P. Potential applications of plant biotechnology against SARS-CoV-2. *Trends Plant Sci.* (2020) 25:635–43. doi: 10.1016/j.tplants.2020.04.009
299. Nakabayashi R, Saito K. Metabolomics for unknown plant metabolites. *Anal Bioanal Chem.* (2013) 405:5005–11. doi: 10.1007/s00216-013-6869-2
300. Li D, Halitschke R, Baldwin IT, Gaquerel E. Information theory tests critical predictions of plant defense theory for specialized metabolism. *Sci Adv.* (2020) 6:eaaz0381. doi: 10.1126/sciadv.aaz0381
301. Bhuiyan FR, Campos NA, Swennen R, Carpentier S. Characterizing fruit ripening in plantain and Cavendish bananas: a proteomics approach. *J Proteomics.* (2020) 214:103632. doi: 10.1016/j.jprot.2019.103632
302. Runfeng L, Yunlong H, Jicheng H, Weiqi P, Qinhai M, Yongxia S, et al. Lianhuaqingwen exerts anti-viral and anti-inflammatory activity against novel coronavirus (SARS-CoV-2). *Pharmacol Res.* (2020) 156:104761. doi: 10.1016/j.phrs.2020.104761
303. Cyranoski D. China is promoting coronavirus treatments based on unproven traditional medicines. *Nature*. (2020). doi: 10.1038/d41586-020-01284-x. [Epub ahead of print].
304. Nguyen-Vo TH, Nguyen L, Do N, Nguyen TN, Trinh K, Cao H, et al. Plant metabolite databases: from herbal medicines to modern drug discovery. *J Chem Inf Model*. (2020) 60:1101–10. doi: 10.1021/acs.jcim.9b00826
305. Johnson SR, Lange BM. Open-access metabolomics databases for natural product research: present capabilities and future potential. *Front Bioeng Biotechnol.* (2015) 3:22. doi: 10.3389/fbioe.2015.00022

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Communication

BRIEF RESEARCH REPORT
published: 07 August 2020
doi: 10.3389/fcomm.2020.00056

COVID-19 Consumer Health Information Needs Improvement to Be Readable and Actionable by High-Risk Populations

Alison Caballero[1]*, *Katherine Leath*[1] and *Jamie Watson*[2]

[1] Center for Health Literacy, University of Arkansas for Medical Sciences, Little Rock, AR, United States, [2] Department of Medical Humanities and Bioethics, College of Medicine, University of Arkansas for Medical Sciences, Little Rock, AR, United States

Health communicators help promote recommended health behaviors by providing accurate, actionable health information that is easy to read and understand. The COVID-19 public health crisis presents a special challenge to clear health communication because some populations most affected by the virus are also at risk for limited health literacy. We collected 28 consumer COVID-19 materials from the internet using popular search engines. We then assessed the materials for readability, understandability, and actionability using validated tools. Aggregate results suggest that the sample of materials was difficult to read and lacked a number of recommended features that promote a readers' ability to understand and act upon the information. We present these findings, their implications for health equity, and their limitations and then suggest ways to improve future health communication about time-sensitive infectious diseases.

Keywords: health literacy, public health, COVID-19, consumer health information, health education, health behavior, health equity

OPEN ACCESS

Edited by:
Rukhsana Ahmed,
University at Albany, United States

Reviewed by:
Victoria Team,
Monash University, Australia
Maria Beatriz Torres,
Mercyhurst University, United States

***Correspondence:**
Alison Caballero
bacaballero@uams.edu

Specialty section:
This article was submitted to Health Communication, a section of the journal Frontiers in Communication

Received: *12 May 2020*
Accepted: *03 July 2020*
Published: *07 August 2020*

Citation:
Caballero A, Leath K and Watson J (2020) COVID-19 Consumer Health Information Needs Improvement to Be Readable and Actionable by High-Risk Populations. Front. Commun. 5:56. doi: 10.3389/fcomm.2020.00056

INTRODUCTION

COVID-19 is a respiratory illness caused by the novel coronavirus SARS-CoV-2 (Lai et al., 2020), identified in late December of 2019. On March 11, 2020, the World Health Organization (WHO) declared a pandemic[1]. On March 31, 2020, the WHO reported a worldwide burden of 750,890 confirmed cases and 36,405 deaths[2]. Also on March 31, 2020, the Institute for Health Metrics and Evaluation projected 83,967 deaths from COVID-19 in the US alone by August 1[3]. As of June 7, 2020, at the time of this writing, there have been 109,901 US deaths[4].

[1]World Health Organization. (2020). *WHO Director-General's opening remarks at the media briefing on COVID-19-11 March 2020*. Available online at: https://www.who.int/dg/speeches/detail/who-director-general-s-opening-remarks-at-the-media-briefing-on-covid-19-11-march-2020 (accessed April 16, 2020).

[2]World Health Organization. (2020). *Coronavirus disease 2019 (COVID-19) Situation Report-71*. Available online at: https://www.who.int/docs/default-source/coronaviruse/situation-reports/20200331-sitrep-71-covid-19.pdf?sfvrsn=4360e92b_4 (accessed April 14, 2020).

[3]The Institute for Health Metrics and Evaluation. (2020). *Main updates on US COVID-19 predictions since March 30, 2020*. Available online at: http://www.healthdata.org/sites/default/files/files/Projects/COVID/Estimation_update_033120.pdf (accessed April 16, 2020).

[4]Centers for Disease Control and Prevention. (2020). *Cases in the U.S.* Available online at: https://www.cdc.gov/coronavirus/2019-ncov/cases-updates/cases-in-us.html (accessed June 2, 2020).

One of the WHO's strategic objectives to respond to this pandemic included public communication about disease risk along with details to counter misinformation[2]. Many organizations have posted information about COVID-19 online in an effort to educate the public about the evolving situation. Likewise, the public is seeking information about the pandemic. A study conducted by the Pew Research Center in late March 2020 found that 7 in 10 US adults had used the internet to learn about COVID-19[5].

In their communications, public health authorities frequently include actions that community members can take to limit the spread of COVID-19 (e.g., properly washing hands and staying home if ill). To follow public health guidance, consumers must be able to read and understand instructions, but this is a challenge for most adult Americans. Only 12 out of 100 have proficient health literacy skills (Kutner et al., 2006). Being able to read, or general literacy, is important to health literacy, which comprises a larger set of skills. Beyond the ability to read prose narrative, health literacy skills encompass information-seeking, interacting with forms and other documents, and the ability to use numbers to improve health.

Populations at risk for limited health literacy include those who are older and those from minority groups (Kutner et al., 2006). Compared with 12 out of 100 adults in the general population with proficient health literacy, only three out of 100 adults over age 65 fall into this category. Similarly, most racial and ethnic minorities fare worse than their white peers. Among African Americans and Hispanics, just two and four out of 100, respectively, demonstrate proficient health literacy skills (Kutner et al., 2006).

Of note, the same populations are at increased risk for COVID-19 infection and death (Mueller et al., 2020). Adults over 65 make up ∼80% of COVID-19 hospitalizations. Weekly reports continue to show greater numbers of COVID-19 deaths among older adults[6]. Many states are reporting that African Americans experience disproportionate rates of positive COVID-19 cases and death (Abrams and Szefler, 2020)[7]. As an example, an April 2020 report showed that while African Americans comprised only about 30% of the population of the state of Louisiana, they accounted for closer to 70% of the state's COVID-19 deaths (Yancy, 2020). And in Baltimore, where Hispanics comprised 5% of the population, they accounted for 12% of the COVID-19 patient population[8]. A May 29, 2020 report stated that rates of hospitalizations for African Americans were 4.5 times that of whites, and rates for Hispanics were 3.5 times that of whites[9]. Because these populations are at increased risk for COVID-19 and its complications and are also likely to be limited by health literacy, clear communication with them about COVID-19 is of critical importance.

While often described as a measure of individual capacity, health literacy is influenced not only by individual characteristics, but by the demands or complexities of the health information itself[10]. To address limitations in the health literacy skills of consumers, communicators can follow recommended practices to produce health information that is readable, understandable, and actionable[11,12].

Our team used validated formulas and tools to formally assess a sample of highly visible online COVID-19 materials retrieved during late March 2020 to determine how readable, understandable, and actionable they were. The results point to a number of techniques that could be used to improve current and future messaging and to better engage individuals in public health behaviors that limit the spread of infectious diseases.

MATERIALS AND METHODS

The purpose of this study was to evaluate online COVID-19 information intended for and easily accessible by the general public.

To locate the content most likely to be viewed by consumers, we used three top search engines (Google, Yahoo!, and Bing)[13] and searched for content using the "incognito" method to prevent previous internet search history from affecting the search results. During the week of March 23, 2020, we entered four terms into each search engine: coronavirus, covid-19, covid19, and covid 19. Within each set of search results, we chose the top six web site links for further review. We excluded all sponsored or promoted content and content from news outlets. In addition to helping avoid bias that may be found in these materials, our selected assessment tool is designed to assess educational materials rather than news or advertisements.

Of the top 72 links initially identified, we removed 44 duplicates. We followed the remaining 28 links to their respective

[5]Pew Research Center. (2020). *Americans turn to technology during COVID-19 outbreak, say an outage would be a problem*. Available online at: https://www.pewresearch.org/fact-tank/2020/03/31/americans-turn-to-technology-during-covid-19-outbreak-say-an-outage-would-be-a-problem/ (accessed April 14, 2020).

[6]Centers for Disease Control and Prevention. (2020). *Weekly Updates by Select Demographic and Geographic Characteristics*. Available online at: https://www.cdc.gov/nchs/nvss/vsrr/covid_weekly/index.htm (accessed June 2, 2020).

[7]American Association of Retired Persons. (2020). *Blacks, Hispanics Hit Harder by the Coronavirus, Early U.S. Data Show*. Available online at: https://www.aarp.org/health/conditions-treatments/info-2020/minority-communities-covid-19.html (accessed June 2, 2020).

[8]The Baltimore Sun. (2020). *Latinos disproportionately hurt by coronavirus in Maryland, Baltimore and among Johns Hopkins patients*. Available online at: https://www.baltimoresun.com/coronavirus/bs-md-covid-latinos-20200512-s3cjb6swbbfofmmfg7afmj3zw4-story.html (accessed June 2, 2020).

[9]Centers for Disease Control and Prevention. (2020). *COVIDView Weekly Summary, Key Updates for Week 22, ending May 30, 2020*. Available online at: https://www.cdc.gov/coronavirus/2019-ncov/covid-data/covidview/index.html (accessed June 2, 2020).

[10]Office of Disease Prevention and Health Promotion. (2020). *Health Literacy—Healthy People 2020*. Available online at: https://www.healthypeople.gov/2020/topics-objectives/topic/social-determinants-health/interventions-resources/health-literacy (accessed April 16, 2020).

[11]Plain Language Action and Information Network (PLAIN). (2020). *Federal plain language guidelines*. Available online at: https://plainlanguage.gov/guidelines/ (accessed April 16, 2020).

[12]Agency for Healthcare Research and Quality. (Content last reviewed May 2015). *Tip 6. Use Caution With Readability Formulas for Quality Reports*. Available online at: https://www.ahrq.gov/talkingquality/resources/writing/tip6.html (accessed April 20, 2020).

[13]StatCounter GlobalStats. (2020). *Search Engine Market Share Worldwide—April 2020*. Available online at: https://gs.statcounter.com/search-engine-market-share (accessed May 8, 2020).

web sites. If the material that appeared at the landing page met our inclusion criteria, we included it in our assessment. If it did not, we identified the next available appropriate material within the site, using a left-to-right and top-to-bottom approach. Inclusion criteria were met if the material was clearly directed at the general public and if it was at least 100 words in length. The software we used for readability assessments includes a cautionary note that samples smaller than 100 words do not produce valid readability results. The majority of final materials assessed were posted by public health entities at the state, national, and international levels.

Once the appropriate content was selected for study, we assessed the materials for readability, understandability, and actionability using standardized processes to promote interrater reliability. To assess readability, two trained staff cleaned each material (e.g., removed bullets and extraneous punctuation) and used Seven Formulas software (Micro Power & Light Co., Dallas, TX, USA) to generate results from three validated formulas: Flesch-Kincaid (Kincaid et al., 1975), SMOG (McLaughlin, 1969), and Fry Graph (Fry, 1968). We averaged the scores from the three formulas to arrive at a mean readability score for each material, and used those to arrive at a mean readability score for the entire sample. Further, we categorized each material and the sample into the "easy," "average," or "difficult" level[12].

Recognizing the limitations of readability assessments to judge how understandable or actionable a material is (McGee, 2010), we used the Patient Education Materials Assessment Tool for Print Materials (PEMAT-P) (Shoemaker et al., 2013) to assess those domains. The PEMAT-P includes 17 items to assess understandability and 7 items to assess actionability and produces separate percentage scores for each area.

In accordance with the PEMAT User's Guide, two reviewers independently assessed each material using the PEMAT scoring sheet. For each item, each user assigned a score of 0 (disagree), 1 (agree), and where indicated by the User's Guide, N/A (not applicable). After independently reviewing each material and scoring it across the 24 items, pairs of reviewers met to review and discuss scores. Where reviewers did not agree on an item, the User's Guide was consulted as needed, reviewers looked at the material together, and after discussion arrived at consensus on a final score for each of the 24 items. Results were entered into an Excel spreadsheet, and authors used Excel features to generate mean scores for readability, understandability, and actionability across materials.

RESULTS

Key Findings: Readability

Using the Flesch-Kincaid, SMOG, and Fry readability formula results obtained from Seven Formulas software, we calculated the mean readability score across the 28-material sample at grade 10, which places the mean in the "difficult" category. Of the 28 selections tested, only 2 (7%) were assessed as "easy" (grade 6 or below). Another 10 (36%) were in the "average" range (grades 7 to 9), and the majority (16, or 57%) were assessed as "difficult" (grade 10 or above).

Key Findings: Understandability and Actionability

The PEMAT-P includes 17 items to assess understandability and seven to assess actionability, and it produces a percentage score for each domain. The higher the percentage score, the more understandable or actionable the material is. Our study revealed some important strengths and weaknesses of online consumer information about COVID-19. Results for selected PEMAT-P items are presented in **Table 1** along with examples to illustrate each of these concepts. The number of materials scored for each item varied as not all PEMAT-P items were relevant to all materials.

The mean score for understandability across all materials was 70%. Overall, the sample scored well on the items related to quantitative expression. Most materials (92%) presented numbers clearly (e.g., used whole numbers rather than fractions or decimals), and all (100%) avoided requiring readers to perform calculations. Another strength is that 75% of materials presented the content in a logical sequence; that is, in the order in which readers would expect.

With respect to actionability, the mean score across the sample was 79%. Almost all materials (93%) included at least one action readers could take, and 64% addressed readers directly when giving instructions.

Unfortunately, there were also several concerning deficiencies in many of these materials. With respect to helping the public understand relevant information, just over half (58%) of the materials made the purpose of the material completely evident. Clearly stating the purpose in the title or introductory text tells a reader whether they are the intended audience and the essence of what they should learn by reading it.

We also observed that many materials failed to use plain language in their word choices. Plain language refers to writing that a reader can understand the first time they see it (Sunstein, 2011). The PEMAT includes two items to assess word choice: one to determine if medical jargon was used unnecessarily or without being defined and another to determine the degree to which common, everyday terms were used (e.g., "used" rather than "utilized"). A majority (57%) failed to meet one or both of these standards.

Fewer than half (39%) of the materials included visual images when such an image would have helped readers understand the information. User guidance from the PEMAT-P directs scorers to reflect a negative (disagree) score when the scorer can identify at least one image that, in included, would improve the likelihood of the reader understanding it. For example, we noted many references to maintaining a 6-foot space between persons. In our experience, many adults struggle to derive value from mathematical concepts such as measurements; thus, this is a concept that would likely be better understood with the addition of a visual image.

Although almost all materials included at least one action a reader could take, fewer than two-thirds (61%) included manageable, explicit steps to act on the instructions. However, for several of those that did include an explicit step, the step was simply to click a link to a different website or document.

TABLE 1 | Selected PEMAT-P scores and examples.

PEMAT-P item	Materials that met the standard number (percent)	Explanations and examples
Understandability (Strengths)		
The material does not expect the user to perform calculations ($n = 28$)	28 (100%)	None of the materials required the reader to add, subtract, multiply or divide, or perform any other mathematical operation.
Numbers appearing in the material are clear and easy to understand ($n = 27$)	25 (92%)	Material should only use numbers when needed and when they are used they should be clear and easy to understand. • Wash your hands with soap and water for at least 20 seconds • 15 days to slow the spread • Avoid social gatherings in groups of more than 10 people.
The material presents information in a logical sequence ($n = 28$)	21 (75%)	Information in these materials were presented in a logical order with the most important information first and similar information grouped together.
Understandabilty (Weaknesses)		
The material uses visual aids whenever they could make content more easily understood (e.g., illustration of healthy portion size) ($n = 28$)	11 (39%)	Many materials would benefit from the use of a picture to show the reader: • How far apart six feet is (a commonly recommended distance to keep between themselves and others) • Samples of appropriate cleaning products and their labeling
The material uses common, everyday language ($n = 28$)	13 (46%)	Examples of technical and likely unfamiliar words found in materials: • Acceleration • Acquired • Contaminated • Continually • Novel • Produced • Sustainably
The material makes its purpose completely evident ($n = 28$)	16 (58%)	Several materials failed to include a title or text upfront to tell the reader at a glance what the material is about.
Medical terms are used only to familiarize the audience with the terms. When used, medical terms are defined ($n = 28$)	18 (64%)	Examples of medical terms used but not defined: • Acute • Cardiovascular disease • Chronic • Infectious diseases • Respiratory hygiene • Saliva
Actionability (Strengths)		
The material clearly identifies at least one action the user can take ($n = 28$)	26 (93%)	Specific actions for the reader to take to keep from getting the virus were included in most materials. Examples included washing hands and staying home when ill.
Actionability (Weaknesses)		
The material breaks down any action into manageable, explicit steps ($n = 28$)	17 (61%)	Examples of actions recommended but not broken down into manageable steps and thus not fully actionable: • Clean and disinfect surfaces (without steps on how to do so) • Seek medical care (with no guidance as to whether to call primary care, urgent care, specialty care, or emergency care)
The material addresses the user directly when describing actions ($n = 28$)	18 (64%)	Example of failing to say "you" or begin a directive with an action verb: • The Department of Public Health recommends that people who have returned from traveling outside the state for business or vacation voluntarily self- isolate for 14 days following their return and monitor for fever and other symptoms

DISCUSSION

Public Health Impact

During public health emergencies, the public needs easy access to health information that is clear, meaningful, and actionable. Our results are similar to those of previous assessments of health information (Davis et al., 1990; Stossel et al., 2012; Haller et al., 2019; Prince et al., 2019) and suggest that even during a high-stakes public health threat, deficiencies in the quality of consumer health information are common. This is especially concerning given that people in high-risk categories for limited health literacy (e.g., older adults, people with chronic health conditions, and minorities) are also the people at high risk of experiencing the worst effects of COVID-19.

These results also have implications for public health ethics. Like other health professions, public health is guided by a set ethical principles (Public Health Leadership Society, 2002;

Thomas et al., 2002). These include equity, transparency, and trust. A common concern among public health ethicists is that standard medical models and practices disenfranchise certain populations, including the elderly (Shepherd, 2019), sex and gender minorities (Littlejohn et al., 2019), and racial and ethnic minorities (Thomas, 2019). Consider, for example, advance directives. While the aim of advance directives is to empower autonomy, many living will forms are "blunt" instruments that lack nuanced options for care. And forms that do attempt to provide more options "become increasingly legalistic, lengthy, and difficult to understand" (Shepherd, 2019, p. 186). People who struggle with health literacy are disproportionately disadvantaged by these obstacles.

Further, many such obstacles have led to suspicion and avoidance of well-ness and preventive care among these populations and are often cited as contributing to health inequities (Casagrande et al., 2007; Bauer et al., 2014; Weisz and Quinn, 2018). For example, LaVeist et al. (2009) found that mistrust of health services among African Americans led to significantly more underutilization of health services than their white counterparts.

Recognizing these obstacles places an additional responsibility on public health officials to reduce barriers to care and to rebuild trust within these communities. This means that a deficiency in the readability and actionability of public-facing health information is also a deficiency in responsibility to public health ethics and a missed opportunity to promote health equity. This responsibility is heightened in public health crises, like COVID-19, where populations already at risk for disenfranchisement are also at higher risk of the worst effects of the virus. However, one way to reduce barriers to care is to improve access to accurate health information by addressing the health literacy needs of populations at higher risk of harm from public health emergencies.

Limitations

Our study was limited by several factors. As our intent was to quickly assess and report on a sample of consumer health information to highlight opportunities to improve communication as the pandemic progresses, we selected a brief period in time and a small number of materials to include in our assessment; thus, the materials chosen may not fully reflect the quality of consumer health materials on this topic.

We gathered our data during the week of March 23, 2020, just weeks after the first case of COVID-19 was reported in the US. Given the lack of expert consensus on many of the issues related to COVID-19 at that time, it is possible that some writers of public health information were cautious about how much information and advice to include in the informational materials.

Further, given the disagreement among experts over certain kinds of advisable actions (e.g., whether the general public should wear masks and whether gatherings of more than two people are safe[14, 15, 16]) it is not surprising that actionability among the materials was limited and that explicit advice may have been omitted intentionally.

Further still, as the pandemic progressed quickly, we acknowledge that for those working in all areas of the COVID-19 response, time was of the essence, and there was likely little time to apply standard editing processes or other routine approaches to optimize clarity.

Although we took steps to mitigate filtering biases in our searches (using multiple computers and searching in "incognito" mode), we acknowledge that our searches could have been influenced by our geographic location and the past search histories of users who work in health-related fields.

Finally, while the validated tools we used to assess these materials are considered the best available, they are not without limitations. Readability is not an exact science, and there is some interprofessional disagreement over how precisely to determine whether a word or phrase is understandable to the "average" person. Although we followed the PEMAT-P user instructions for scoring materials, some subjectivity remained. In those cases, we established a group consensus on how to score items. Lastly, a favorable score on the PEMAT-P does not guarantee that the material is of high quality.

Future Studies

Our findings are similar to many other reviews of consumer health information, which raises questions about how health communicators perceive the readability, understandability, and actionability of their own work. As there is currently no research on these perceptions, future studies could collect evidence about those perceptions and then compare them with the results of validated assessments of their work. When perceptions and formal assessments differ, interventions could advocate for the use of validated assessment tools and provide training for health communicators.

Investigators could also determine the degree to which efforts to ensure readable and actionable materials were employed, along with related barriers. This information could help communications teams plan ahead for providing clear health information during future events that require timely outreach to the public.

Further, while COVID-19 is genuinely novel, it is only one of many infectious diseases that health professionals address regularly, including tuberculosis, hepatitis, and Ebola. These illnesses warrant the time and effort required to develop messaging templates that are readable, understandable, and actionable. These templates would then allow for quickly

[14]Quartz Media, Inc. (2020). *Every expert opinion you've heard about wearing masks is right*. Available online at: https://qz.com/1826717/do-masks-protect-against-coronavirus/ (accessed June 7, 2020).

[15]The Atlantic. (2020). *Masks Are a Tool, Not a Symbol. Lives will be lost if Americans allow the culture war to determine whether they cover their face in public*. Available online at: https://www.theatlantic.com/ideas/archive/2020/05/masks-are-tool-not-symbol/611134/ (accessed June 7, 2020).

[16]Global Policy Journal (2020). *Why Experts Disagree on How to Manage COVID-19: Four Problem Conceptions, Not One*. Available online at: https://www.globalpolicyjournal.com/blog/07/04/2020/why-experts-disagree-how-manage-covid-19-four-problem-conceptions-not-one/ (accessed June 7, 2020).

disseminating information about new public health threats. Our findings suggest that those creating such templates should pay special attention to:

- Stating clearly the purpose of the material
- Reviewing the information for plain language
- Replacing or defining and explaining any medical terms
- Using visual aids to make the material easier to understand or the actions easier to take
- Giving explicit, manageable steps for any actions readers are asked to take

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

KL introduced the topic as a study of interest. KL and AC drafted study design with input from JW. KL and JW each drafted a section of the manuscript, reviewed it in its entirety, and approved the final copy. AC drafted remaining sections, edited all sections, and provided oversight to the assessment and writing process. All authors participated in the use of the Patient Education Materials Assessment Tool to formally assess online materials.

FUNDING

Authors nor their institution received any funding in direct support of the work described nor of the preparation of the brief research report, outside of salaries paid to each author by the institution.

ACKNOWLEDGMENTS

Authors acknowledge Maria Ruvalcaba for her work in assessing readability of selected documents, Jennifer Gan for her editing and reference formatting, Scarlett Rice for her support with reference entry, and the UAMS Science Communication Group for their editorial services.

REFERENCES

Abrams, E. M., and Szefler, S. J. (2020). COVID-19 and the impact of social determinants of health. *Lancet Respirat. Med.* 8, 659–661. doi: 10.1016/S2213-2600(20)30234-4

Bauer, G. R., Scheim, A. I., Deutsch, M. B., and Massarella, C. (2014). Reported emergency department avoidance, use, and experiences of transgender persons in Ontario, Canada: results from a respondent-driven sampling survey. *Annal. Emergency Med.* 63, 713–720.e1. doi: 10.1016/j.annemergmed.2013.09.027

Casagrande, S. S., Gary, T. L., LaVeist, T. A., Gaskin, D. J., and Cooper, L. A. (2007). Perceived discrimination and adherence to medical care in a racially integrated community. *J. Gen. Internal Med.* 22, 389–395. doi: 10.1007/s11606-006-0057-4

Davis, T. C., Crouch, M. A., Wills, G., Miller, S., and Abdehou, D. M. (1990). The gap between patient reading comprehension and the readability of patient education materials. *J. Family Practice* 31, 533–538.

Fry, E. (1968). A readability formula that saves time. *J. Reading* 11, 513–516.

Haller, J., Keller, Z., Barr, S., Hadden, K., and Oliphant, S. S. (2019). Assessing readability: are urogynecologic patient education materials at an appropriate reading level? *Female Pelvic Med. Reconstr. Surg.* 25, 139–144. doi: 10.1097/SPV.0000000000000653

Kincaid, J., Fishburne, R., Rogers, R., and Chissom, B. (1975). Derivation of new readability formulas (automated readability index, fog count and flesch reading ease formula) for navy enlisted personnel. *Institute Simulation Training*. 1–39. doi: 10.21236/ADA006655

Kutner, M., Greenburg, E., Jin, Y., Paulsen, C., and White, S. (2006). *The Health Literacy of America's Adults: Results From the 2003 National Assessment of Adult Literacy.* Washington, DC: National Center for Education Statistics. 76.

Lai, C.-C., Shih, T.-P., Ko, W.-C., Tang, H.-J., and Hsueh, P.-R. (2020). Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) and Coronavirus Disease-2019 (COVID-19): the epidemic and the challenges. *Int. J. Antimicrob. Agents* 55:105924. doi: 10.1016/j.ijantimicag.2020.105924

LaVeist, T. A., Isaac, L. A., and Williams, K. P. (2009). Mistrust of health care organizations is associated with underutilization of health services. *Health Services Res.* 44, 2093–2105. doi: 10.1111/j.1475-6773.2009.01017.x

Littlejohn, T., Poteat, T., and Beyrer, C. (2019). "Sexual and gender minorities, public health, and ethics." in *The Oxford Handbook of Public Health Ethics*, *by Tonya Littlejohn, Tonia Poteat, and Chris Beyrer*, eds A. C. Mastroianni, J. P. Kahn, and N. E. Kass (Oxford University Press), 231–44. doi: 10.1093/oxfordhb/9780190245191.013.17

McGee, J. (2010). *Toolkit for Making Written Material Clear and Effective*. Baltimore, MD: Centers for Medicare and Medicaid Services. Available online at: https://www.cms.gov/Outreach-and-Education/Outreach/WrittenMaterialsToolkit/Downloads/ToolkitPart07.pdf (accessed April 20, 2020).

McLaughlin, G. H. (1969). SMOG grading: a new readability formula. *J. Reading* 12, 639–646.

Mueller, A. L., McNamara, M. S., and Sinclair, D. A. (2020). Why does COVID-19 disproportionately affect the elderly? *Aging* 12, 9959–9981. doi: 10.20944/preprints202004.0548.v1

Prince, L. Y., Mears, S. C., Watson, J. C., and Hadden, K. B. (2019). Health literacy evaluation of opioid patient education materials for orthopaedic surgery. *J. Surg. Orthop. Adv.* 28, 232–236. doi: 10.3113/JSOA.2019.0232

Public Health Leadership Society (2002). *Principles of the Ethical Practice of Public Health*. Available online at: https://www.apha.org/-/media/files/pdf/membergroups/ethics/ethics_brochure.ashx (accessed April 17, 2020).

Shepherd, L. (2019). "Aging populations and public health ethics." in *The Oxford Handbook of Public Health Ethics*, *by Lois Shepherd*, eds A. C. Mastroianni, J. P. Kahn, and N. E. Kass (Oxford University Press), 180–91. doi: 10.1093/oxfordhb/9780190245191.013.19

Shoemaker, S. J., Wolf, M. S., and Brach, C. (2013). *The Patient Education Materials Assessment Tool (PEMAT) and User's Guide.* Rockville, MD: US Dept of Health and Human Services, Agency for Healthcare Research and Quality. Avaialble online at: https://www.ahrq.gov/sites/default/files/publications2/files/pemat_guide_0.pdf (accessed April 21, 2020).

Stossel, L. M., Segar, N., Gliatto, P., Fallar, R., and Karani, R. (2012). Readability of patient education materials available at the point of care. *J. Gen. Internal Med.* 27, 1165–1170. doi: 10.1007/s11606-012-2046-0

Sunstein, C. R. (2011). *Final Guidance on Implementing the Plain Writing Act of 2010. Memorandum M-11–15*. Washington, DC: Office of Management and Budget. Available online at: https://www.whitehouse.gov/sites/whitehouse.gov/files/omb/memoranda/2011/m11-15.pdf (accessed April 21, 2020).

Thomas, J. C., Sage, M., Dillenberg, J., and James Guillory, V. (2002). A code of ethics for public health. *Am. J. Public Health* 92, 1057–1059. doi: 10.2105/AJPH.92.7.1057

Thomas, S. B. (2019). "Racial and ethnic disparities as a public health issue." in *The Oxford Handbook of Public Health Ethics*, *by Stephen Thomas*, eds A. C. Mastroianni, J. P. Kahn, and N. E. Kass (Oxford: Oxford University Press), 277–89. doi: 10.1093/oxfordhb/9780190245191.013.26

Weisz, C., and Quinn, D. M. (2018). Stigmatized identities, psychological distress, and physical health: intersections of homelessness and race. *Stigma Health* 3, 229–240. doi: 10.1037/sah0000093

Yancy, C. W. (2020). COVID-19 and African Americans. *JAMA* 323:1891. doi: 10.1001/jama.2020.6548

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 07 August 2020
doi: 10.3389/fimmu.2020.01748

Remodeling of the Immune Response With Aging: Immunosenescence and Its Potential Impact on COVID-19 Immune Response

Lucas Leite Cunha[1], *Sandro Felix Perazzio*[2], *Jamil Azzi*[3], *Paolo Cravedi*[4] and *Leonardo Vidal Riella*[5,6]*

[1] Department of Medicine, Escola Paulista de Medicina, Federal University of São Paulo, São Paulo, Brazil, [2] Division of Rheumatology, Escola Paulista de Medicina, Federal University of São Paulo, São Paulo, Brazil, [3] Schuster Transplantation Research Center, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, United States, [4] Renal Division, Department of Medicine, Icahn School of Medicine at Mount Sinai, New York, NY, United States, [5] Division of Nephrology, Massachusetts General Hospital, Harvard Medical School, Boston, MA, United States, [6] Department of Surgery, Center for Transplantation Sciences, Massachusetts General Hospital, Boston, MA, United States

OPEN ACCESS

Edited by:
Massimo Triggiani, University of Salerno, Italy

Reviewed by:
Sebastiano Gangemi, University of Messina, Italy
Remo Castro Russo, Federal University of Minas Gerais, Brazil

***Correspondence:**
Leonardo Vidal Riella
lriella@mgh.harvard.edu

Specialty section:
This article was submitted to Cytokines and Soluble Mediators in Immunity, a section of the journal Frontiers in Immunology

Received: *20 April 2020*
Accepted: *30 June 2020*
Published: *07 August 2020*

Citation:
Cunha LL, Perazzio SF, Azzi J, Cravedi P and Riella LV (2020) Remodeling of the Immune Response With Aging: Immunosenescence and Its Potential Impact on COVID-19 Immune Response. Front. Immunol. 11:1748. doi: 10.3389/fimmu.2020.01748

Elderly individuals are the most susceptible to an aggressive form of coronavirus disease (COVID-19), caused by SARS-CoV-2. The remodeling of immune response that is observed among the elderly could explain, at least in part, the age gradient in lethality of COVID-19. In this review, we will discuss the phenomenon of immunosenescence, which entails changes that occur in both innate and adaptive immunity with aging. Furthermore, we will discuss inflamm-aging, a low-grade inflammatory state triggered by continuous antigenic stimulation, which may ultimately increase all-cause mortality. In general, the elderly are less capable of responding to neo-antigens, because of lower naïve T cell frequency. Furthermore, they have an expansion of memory T cells with a shrinkage of the T cell diversity repertoire. When infected by SARS-CoV-2, young people present with a milder disease as they frequently clear the virus through an efficient adaptive immune response. Indeed, antibody-secreting cells and follicular helper T cells are thought to be effectively activated in young patients that present a favorable prognosis. In contrast, the elderly are more prone to an uncontrolled activation of innate immune response that leads to cytokine release syndrome and tissue damage. The failure to trigger an effective adaptive immune response in combination with a higher pro-inflammatory tonus may explain why the elderly do not appropriately control viral replication and the potential clinical consequences triggered by a cytokine storm, endothelial injury, and disseminated organ injury. Enhancing the efficacy of the adaptive immune response may be an important issue both for infection resolution as well as for the appropriate generation of immunity upon vaccination, while inhibiting inflamm-aging will likely emerge as a potential complementary therapeutic approach in the management of patients with severe COVID-19.

Keywords: immunosenescence, inflammaging, SARS-CoV-2, COVID-19, immunopathogenesis

INTRODUCTION

In December 2019, a novel coronavirus, severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), was discovered as the causative agent of an outbreak of viral lower-respiratory tract infections centered in Wuhan (China) (1). Since then, SARS-CoV-2 has caused a widespread outbreak of severe acute respiratory syndrome throughout China, with exported cases occurring in other continents, including the United States, in a worldwide pandemic (1). Interestingly, a strong age gradient in the risk of death was observed among patients with coronavirus disease (COVID-19) (2). In this scenario, the remodeling of immune response that is observed among the elderly could be a possible explanation for the higher lethality of COVID-19 noted on this population.

The immune response is dynamically remodeled with aging, a phenomenon denominated as immunosenescence. This phenomenon increases susceptibility to a myriad of clinical conditions such as infections, autoimmune disorders, and malignancies. Recent data had shed light on the physiological aspects of immunosenescence, which is now considered an immune adaptation to the aged microenvironment rather than merely a collapse of the system (3).

Both the innate and adaptive immunity is affected by aging. Some individuals experience a sustained innate immune system activation, inducing proinflammatory cytokines secretion and innate immune cells' recruitment (4). Innate immunity hyperactivation may be detrimental and impair global functionality, causing a clinical phenotype known as frailty syndrome. Frailty syndrome is defined as a state of cumulative decline in several physiological systems with a disproportionate vulnerability to stressor events (5). Frailty syndrome prevalence increases with age, it is multifactorial in etiology, and the physical component of frailty can be objectively assessed by the Fried Frailty Score (Phenotype Score) and the Frailty Index (Deficit Accumulation Index) (6).

Likewise, adaptive immunity remarkably changes as age increases, which can be summarized into two main topics: (1) bone marrow reorganization and hematopoietic stem cell pool differentiation into myeloid lineage, outnumbering lymphoid compartment; and (2) physiological thymic involution, compromising naïve T cells generation. The sum of these two factors can help explain the prior known impairment of the regenerative capacity of lymphocytes compared to myeloid-derived cells in the elderly (7).

Infectious diseases are more prevalent among the elderly. When compared to younger counterparts, the elderly more frequently present with respiratory and urinary tract infections, and those patients usually have a worse prognosis (8, 9). It is possible that the impaired barrier function of mucosae and diminished adaptive immune response (both cellular and humoral) are the reasons for the increased susceptibility to infectious microorganisms among the elderly (10). In addition, the natural killer (NK) cell senescence may affect the homeostasis of the immune system in the elderly, leading to an increased risk of cancer and additional risk of viral infections (11). Lastly, age-related cell dysfunctions leading to an exhausted phenotype are also an important characteristic of the immune system remodeling with aging, which might accelerate tissue damage and disable modulatory mechanisms (12). Herein, we review the state of the art research on senescence-induced immune dysregulation, focusing on innate and adaptive cell functional analysis and its potential impact in viral immune responses, such as in COVID-19.

PHYSIOLOGY OF IMMUNOSENESCENCE AND INFLAMM-AGING

Currently, the concept of immunosenescence refers to a comprehensive remodeling of the immune system and its microenvironment, involving both innate and adaptive compartments that occur with aging (13, 14). Many physiological phenomena have been proposed to explain the immune response remodeling over time, including chronic exposure to antigens, impaired telomerase activity, mitochondrial dysfunction, defective autophagy, endoplasmic reticulum stress, defective ubiquitin/proteasome system, and age-related changes in the composition of gut microbiota (15–18). Probably, a melting pot of diverse factors differently contributes to the final phenotype of the adapted and experienced immune system, named immunosenescence.

Aging of the immune system is characterized by an imbalance between stimulatory and regulatory mediators, such as cytokines and acute phase reactants, toward a sub-clinical chronic proinflammatory state called inflamm-aging. Inflamm-aging is thought to be caused by a low-grade inflammation secondary to continuous antigenic stimulation (19), whose source may be exogenous, like a pathogenic microorganism infection (20, 21), or endogenous (15–18), like post-translational-modified macromolecules (15). Population studies incorporate the notion that the immune response depends on environmental exposure and how it interacts with endogenous variables. In fact, diet, exercise, xenobiotic exposure, and other environmental factors may epigenetically affect the metabolic health of immune cells (22). Lifestyle factors, such as exercise and favorable dietary habits, positively affect the immune system (22), while poor nutrition and reduced muscle mass may predispose an individual to a proinflammatory condition (23).

Innate Immune Response and Inflamm-Aging

Age-related remodeling of innate immunity modifies the homeostasis of NK cells, neutrophils, and monocytes/macrophages (24). NK cells from the elderly exhibit impaired perforin release upon stimulation and granule exocytosis (25, 26). It reduces the elimination of senescent cells, which, in turn, promotes senescent cell accumulation in aged tissue. Moreover, aging reduces the frequency of circulating NK $p46^+$ cells, a modulatory cell subset involved in the resolution of inflammation and elimination of effector cells (27, 28).

Neutrophils and macrophages are classically classified as part of innate immunity and possibly comprise the most important effector cells against bacterial infections. It is thought that age

is accompanied by a decline in production and secretion of most chemokines, including those responsible for neutrophil and monocyte chemoattraction (29). The absolute number of neutrophils seems to be maintained while the number of monocytes increase with age (30, 31). However, the function of these cells may be impaired among the elderly (32). The final consequence is that the delayed resolution of inflammation may be associated with age-related remodeling of neutrophils and macrophages (29).

In addition to their phagocytosis' capabilities, neutrophils are capable of releasing a mesh-like structure under specific circumstances, called neutrophil extracellular traps (NET), in an attempt to physically delimitate the pathogenic agent, mainly microorganisms, and facilitate its contact with microbicidal peptides and enzymes (33). NET is composed of a decondensed chromatin meshwork imbedded with granule proteins with anti-microbial properties. NET may also work as a physical path for immune cell migration to the inflammatory site (34). Neutrophil function is impaired in both animal models and humans with aging. Hazeldine et al. (35) observed that older adults have less IL-8 production, LPS-induced NET release, and cell migration compared to younger counterparts, probably secondary to an impaired signal transduction. Microbicidal killing, phagocytic activity (36), and degranulation capacity (37) of neutrophils are also reduced in the elderly. In addition, the same group investigated the migration pattern of neutrophils obtained from older compared to young adults. They observed that neutrophils from older subjects migrated with less accuracy than those from younger subjects. By inaccurately meandering among healthy tissues, neutrophils from the elderly inadvertently release more neutrophil proteinase that may contribute to tissue damage and systemic inflammation.

Reactive oxygen species (ROS) are free radicals produced after oxidative bursts in phagosomes, which are pivotal for the microbicidal function of phagocytes (38). In fact, ROS do not just directly contribute to the bacterial clearance, but additionally can trigger NET formation. The free radical ROS production by neutrophils in older adults is decreased (39, 40). Interestingly, polymorphonuclear leucocytes from the elderly are less capable of modulating the triggering receptor expressed on myeloid cell-1 (TREM-1)-induced oxidative bursts, suggesting that TREM-1 signal transduction altered with aging may be one of the mediators of the decrease in microbicidal potential of innate immune cells in older adults (41).

Animal models of premature immunosenescence have also shed some light into age-related remodeling of the immune system. Guayerbas et al. (42) described a mouse model of premature immunosenescence based on the demonstration of early decline of immune parameters and behavioral tests in Swiss outbred mice. Mouse model-derived peritoneal leukocytes exhibited reduced proliferative response, impaired NK activity, and increased *in vitro* TNF-alpha production compared to control mice (42). In addition, mouse model-derived macrophages of premature models were less functional with a striking loss of microbicidal activity (43).

The mice model of premature immunosenescence was refined and new models were developed as well (44, 45). Apparently, the key phenomenon are the oxidative and inflammatory stresses, which, not without reason, are associated with several non-communicable chronic diseases prevalent among the elderly (44, 46). In fact, spleen and thymus cells from prematurely immunosenescent mice models have decreased antioxidant defenses and significantly increased oxidants and pro-inflammatory cytokines production (44–46). Interestingly, the antioxidant vs. oxidant imbalance observed in prematurely immunosenescent mice was similar to the one observed in old wild-type animals (44, 47). Hence, lab tests determining the oxidative burst profile of phagocytes (e.g., nitro blue tetrazolium test, dihydrorhodamine oxidation, O_2^- and $H_2O_2^-$ production by chemoluminescence, etc.) may be useful for assessing inflamm-aging features (4).

The state of chronic inflammation has to be counter-balanced by anti-inflammatory molecules (48). When not under control, the low-grade inflammation loses its defense role and turns into a damaging state to the whole organism (49). The practical consequence is that inflamm-aging is deleterious to human health, predicts frailty, and is associated with higher mortality rates (50–52).

Remodeling of the Adaptive Immune System With Aging

Remodeling of the adaptive immune response also occurs with aging. Thymic involution and hematopoietic stem cell insufficiency play important roles in immunosenescence of adaptive immunity (53). In general, elderly individuals are less able to respond to neo-antigens, due to the reduction of new thymus-emergent T cells, though homeostatic proliferation can partially sustain the richness of the TCR repertoire (54, 55). Moreover, peripheral T cells usually present a reduced absolute number in aged individuals with an inverted CD4:CD8 ratio and expansion of terminally differentiated effector memory T cells (56, 57), associated with impaired proliferation ability, telomerase activity, and intracellular signaling (58, 59). Furthermore, most adult regulatory T lymphocytes are a terminally differentiated highly suppressive apoptosis-prone population with a limited capacity for self-renewal (60). This finding might explain, at least in part, the occurrence of age-related autoimmune conditions. In addition, the imbalance between innate and adaptive immunity may disturb the fine regulation of the effector immune response, leading to a severe acute pro-inflammatory state that may lead to organ rejection in transplanted patients (61, 62).

While naïve T and B cells become dysfunctional with aging, memory T and B cells' function is relatively maintained (63–65). In fact, naïve T lymphocytes obtained from the elderly present impaired cell binding of the immune synapse (66), reduced signal transduction (67), dysregulation of cytoskeletal function (68), defective protein glycosylation and activation (69), and insufficient IL-2 production (70).

Some authors advocate that age-related T-cell dysfunction is different from T cell exhaustion, a state of low cell responsiveness mediated by chronic conditions, such as viral infections and malignancies (**Figure 1**) (71). Constant antigen stimulation

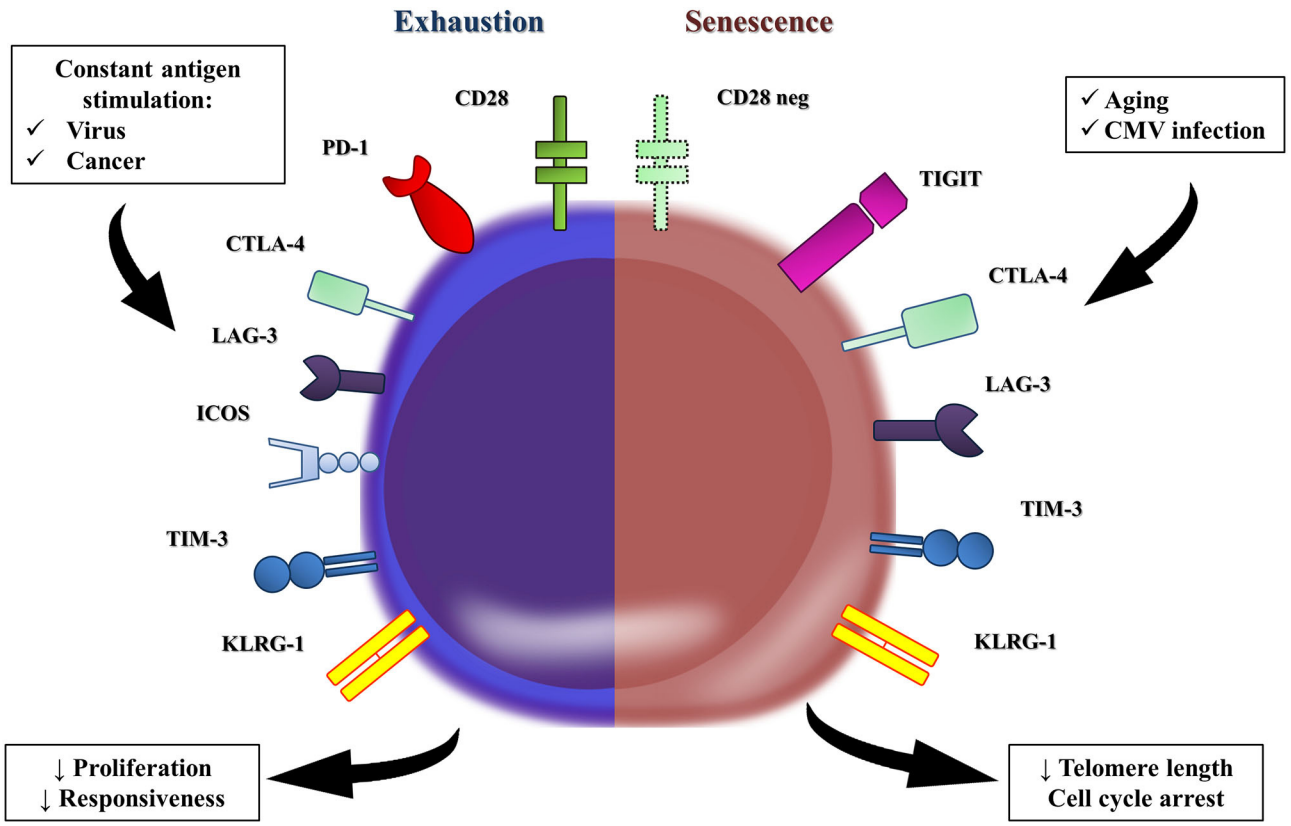


FIGURE 1 | T cell exhaustion vs. T cell senescence. In conceptual terms, the T-cell dysfunction observed in the elderly is different to the one reported as T-cell exhaustion. Persistent viral and cancer stimulation leads to the remodeling of many T cells, which upregulate the expression of co-inhibitory receptors (e.g., PD-1, CTLA-4, LAG-3, ICOS, Tim-3, and KLRG-1), all of them hallmarks of T cell exhaustion. The co-inhibitory receptors downregulate the TCR-stimulated intracellular signal, and T-cells become hyporesponsive and develop responsiveness impairment. However, the immunosenescence is marked by similar levels of PD-1 and TIM-3 and tiny elevations of CTLA-4 and LAG-3 in T cells from the elderly compared to those in younger groups. T-cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif (ITIM) domain (TIGIT) is a co-inhibitory receptor that is expressed on senescent T cells, which exhibited a marked terminal differentiated phenotype. Interestingly, TIGIT-positive T cells from the elderly seems to retain some proliferative capacity, but produced significantly lower amounts of TNF-alpha, IFN-gamma, and IL-2.

progressively exhausts T cells by gradually upregulating the expression of inhibitory checkpoint receptors (e.g., PD-1, CTLA-4, LAG-3, ICOS, Tim-3, and KLRG-1) on $CD4^+$ T cells (72), which, in turn, downmodulate TCR-induced intracellular signaling (73). Interestingly, despite this conceptual difference between immunosenescence and T cell exhaustion, most of those cell exhaustion surface hallmarks are observed on dysfunctional immunosenescent cells, suggesting that these two phenomena share many mechanisms (54).

Exhausted T-cells accumulate over time (67, 74–77). Shimada et al. (74) demonstrated both gene and protein hyper expression of PD-1 and CTLA-4 in cells from old male C57BL/6 mice compared to young controls. Most $PD\text{-}1^+$ T cells were quiescent and presented an anergic effector memory phenotype with impaired proliferative response to mitogens (74). Similarly, Lee et al. (76) reported the accumulation of $Tim\text{-}3^+$ murine T cells with impaired proliferative capacity with aging.

Literature discussing T cell exhaustion and immunosenescence in humans is scarce, though. Song et al. (77) described an elevated number of $TIGIT^+$ $CD8^+$ T cells from old adults, another hallmark of cell exhaustion apparently associated with immunosuppressant features in neoplasm or chronic infection mouse models (78, 79). $TIGIT^+$ $CD8^+$ T cells from old individuals seem to retain a proliferative capacity, although they impaired TNF-alpha, IFN-gamma, and IL-2 *in vitro* production and increased susceptibility to apoptosis (77). Therefore, we hypothesize that evaluation of the proliferative response to mitogens and *in vitro* cytokine production may be indirect ways to assess age-related remodeling of the immune system.

In regards to B cell compartment, vaccine trials suggest that B cell repertoire abridge over time, foremost observed in frail patients (80, 81). In addition, B cells from the elderly present both impaired antibody production and class switch recombination (82). Class switch recombination and immunoglobulin somatic hypermutation are crucial for humoral immune response and occur in mature B cells mediated by activation-induced cytidine deaminase, amongst other mediators (82, 83). Similarly, activated B cells from old mice have less activation-induced cytidine

deaminase expression and reduction of class switched antibodies (84, 85). Interestingly, *in vivo* activated $CD4^+$ T cells from old-aged individuals showed increased dual-specific phosphatase 4 (DUSP4) transcription, which, in turn, negatively correlated with antigen-specific B cells' expansion. Silencing of DUSP4 restored $CD4^+$ T cell-induced B-cell differentiation, suggesting that B cell dysfunction observed with aging is T cell-dependent. **Table 1** summarizes the main physiologic modifications of the immune system in the elderly.

IMMUNE RESPONSE, IMMUNOSENESCENCE, AND COVID-19

Coronaviruses are a large family of viruses that cause upper and lower-respiratory tract illnesses in humans. SARS-CoV-2 is transmitted predominantly via respiratory droplets. Clinically, patients frequently present with fever, cough, myalgia, and fatigue (95). In a subset of patients, mainly elderly individuals, SARS-CoV-2 was shown to lead to bilateral pulmonary diffuse alveolar damage that may progress to acute respiratory distress syndrome (96, 97). Following the pulmonary phase, patients with poor outcome frequently evolve a life-threatening cytokine storm syndrome, characterized by bursts of pro-inflammatory cytokines and chemokines in the serum (96, 98). The uncontrolled systemic inflammation causes endothelial injury and activation of coagulation cascade. The consequence is an explosive process of disseminated intravascular coagulation and consumption of coagulation factors that leads to organ damage and death.

Innate Immune Response and COVID-19

The innate immune response is the first level of response in the detection and clearance of a viral infection. In SARS-CoV-2, the spike protein (S) mediates the attachment, fusion, and entry of the virus in human cells (99). The protein S strongly binds to angiotensin-converting enzyme 2 receptor leading to the attachment of the virus to the host cell (99). The successful entry needs the priming of the S protein by TMPRSS2, a human cellular serine protease (100). Once in the host cell, SARS-CoV-2 can be detected by macrophages, which orchestrate the production of a pro-inflammatory microenvironment that inhibits viral replication, stimulates adaptive immunity, and recruits other immune cells to the site of infection.

Macrophages from elderly lungs may have a more pronounced production of IL-6 and other pro-inflammatory cytokines in response to stimuli (101). It is possible that IL-6 has a critical role in the immune response of the elderly that mounts against SARS-CoV-2 (102). IL-6 helps the differentiation of Th17 lymphocytes, but inhibits the production of Interferon-γ, which is necessary for the activation of CD8+ cells (102). In addition, IL-6 contributes to a pro-inflammatory microenvironment at the lung that impacts the integrity of the air-blood barrier (103). Patients with severe COVID-19 have a higher IL-6/Interferon-γ ratio than those who present with a moderate disease, which could be related to the cytokine storm leading to lung injury (104–107). Indeed, patients with severe COVID-19 frequently have lower absolute numbers of Interferon-γ producing CD4+ T cells compared to patients with moderate disease (108). Then, when patients with COVID-19 enter the immune dysregulation phase, the increase in IL-6 leads to a relative immunoparalysis that may impair the clearance of SARS-Cov-2 (98). Elderly patients with COVID-19 often present with a severe dysregulation of pro-inflammatory cytokines, such as IL-6 and IL-1 β, which may result in worse outcome (105). Drugs that uncouple IL-1β/IL-1R signaling (anakinra) or IL-6/IL-6R signaling (tocilizumab) may have an immunomodulatory potential and are hypothesized to attenuate the dysfunctional immune response during the hyperinflammatory phase of COVID-19 (98, 109). In fact, some reports suggest that infusion of anakinra (109, 110) and tocilizumab (111) may improve the disease course in patients with severe COVID-19 presentation.

Neutrophils have traditionally been considered the primary immune cells active in the defense against bacterial infections. More recently, neutrophils' role in viral infection has emerged based on observations of its correlation with viral infection severity and neutrophils' biological ability to recognize viruses (via viral PAMPs) and respond to them with specific effector functions (112). Patients with severe COVID-19 more frequently present with a high neutrophil-to-lymphocyte ratio (113), in part driven by the relative lymphopenia or lymphocyte exhaustion. In addition, patients with severe COVID-19 are more susceptible to a greater burst of systemic inflammation and secondary bacterial infection that can lead to the increment of neutrophils. It is unclear if changes in neutrophils are only a reflection of the overall immune activation in COVID-19 or if they play a direct pathogenic role. Lastly, NK cells are less functional in the elderly, and studies have shown that severe COVID-19 patients have further depleted peripheral NK cell counts in comparison with mild cases and healthy controls (114–116). Generally, NK cells are capable of recognizing infected cells and of triggering direct cell toxicity. Further studies are needed to clarify how SARS-CoV2-infected cells interact with NK cells and if any apoptosis or downmodulation occurs and prevents the effective elimination of infected cells.

The airway epithelium is a physical barrier to pathogens (117). The integrity of the air-blood barrier is essential for the maintenance of lung homeostasis and represents an important branch of innate immunity (118). The invasion of the airway epithelial by SARS-CoV-2 may break the barrier integrity, triggering a vicious cycle of inflammation and tissue injury that is more pronounced among the elderly (119). Presumably, the same remodeling process that occurs in the immune system also happens at the lung microenvironment with aging (120). Data from animal models suggest that senescent lungs are more susceptible to settle a pro-inflammatory response when injured (121). In fact, bronchoalveolar lavage obtained from elderly patients with acute respiratory distress syndrome present with higher pro-inflammatory cytokine levels when compared to younger counterparts, suggesting that the lung may represent a small fraction of the inflamm-aging that occurs at the systemic level (122). This local phenomenon may help to explain why elderly patients with COVID-19 are more susceptible to a

TABLE 1 | Summary of the age-related physiologic modifications of the immune system.

Cell	Immune response	Aging functional impairment	Clinical impact	References
NK	Innate	• ↓ Perforin degranulation	• Wound healing • Susceptibility to infection • Susceptibility to cancer	(26)
Neutrophil	Innate	• ↓ Phagocytosis • ↓ ROS production • ↓ Intracellular killing • ↓ NET • ↓ Migration	• Wound healing • Susceptibility to infection	(35, 86, 87)
Basophil	Innate	• Delayed degranulation	• Susceptibility to helminth infection • Decrease in allergy parameters	(88–90)
Eosinophil	Innate	• Delayed degranulation • ↓ Superoxide production	• Susceptibility to helminth infection • Decrease in allergy parameters	(88, 91)
T cell	Adaptive	• ↓ Repertoire • Relative decrease of naïve T cells • Relative increase of memory T cells	• Impaired response to vaccination • Susceptibility to infection	(54–60)
B cell	Adaptive	• ↓ Repertoire of antibodies	• Impaired response to vaccination • Susceptibility to infection	(80–82, 84, 85)
Dendritic cells	Adaptive	• ↓ Antigen presentation • ↓ Tolerant response	• Susceptibility to skin and mucosal infection • Susceptibility to autoimmune disorder • Impaired response to vaccination • Transplant rejection	(31, 92–94)

Arrow means diminished.

more severe lung injury that implies loss of lung function and respiratory failure (123).

Adaptive Immune Response and COVID-19

The initial inflammation in COVID-19 is propitious to the activation and differentiation of CD4+ and CD8+ T cells. The ideal final output is the development of an effective and specific immune response, involving both the production of anti- SARS-CoV-2 antibodies and the deployment of a large number of viral-specific cytotoxic lymphocytes that will ultimately eliminate the virus and achieve clinical recovery. In fact, when compared to severe H7N9 disease, reduced pro-inflammatory cytokines and chemokines were found in COVID-19 patients with good prognosis, reinforcing the idea that adaptive immunity is a key factor for a favorable outcome (124).

Thevarajan et al. (124) described a kinetic of the immune response in a 47-year-old woman with COVID-19 who presented a favorable outcome. They evidenced a persistent increase in antibody-secreting cells, follicular helper T cells, activated CD4+ and CD8+ T cells, and immunoglobulin M (IgM) and IgG antibodies that bound to SARS-CoV-2. The peak of both antibody-secreting cells and follicular helper T cells was markedly higher in the patient compared to healthy controls and both cell subsets were persistently increased during convalescence (day 20). The experience from the SARS epidemic of 2003 showed that convalescent SARS patients present with neutralizing antibodies against S protein (125). The sera stored from convalescent patients from the SARS epidemic of 2003 can cross-neutralize the S protein-mediated SARS-CoV-2 entry in patients with COVID-19 (100). This data raises the possibility that the S protein could be an important antigen to vaccine protocols. In fact, in analogy to the SARS epidemic of 2003, convalescent patients with SARS may present IgG and neutralizing antibodies peaking at 4 months after the disease and detectable up to 2 years afterwards, suggesting that memory B cells can be elicited during coronavirus infection (125).

Cellular immune response may play a critical role in the adaptive immune response in patients with COVID-19. Thevarajan et al. (124) observed the emergence and rapid increase in activated CD8+ T cells at days 7–9 after infection preceded the resolution of symptoms of one young patient with a good prognosis. Conversely, elderly patients and those requiring intensive care unit support presented a dramatically reduced number of CD4+ and CD8+ T cells (126). Lower total amounts of T cells, CD4+, and CD8+ T cells negatively correlated with patient survival (126). Diao et al. (126) noted that T cell absolute counting were negatively correlated to serum IL-6, IL-10, and TNF-α concentration in patients with COVID-19, suggesting that the failure of the adaptive immune response and the increase of pro-inflammatory cytokine may be associated with worse survival. It is also possible that increased IL-6 leads to a reduction in $CD4^+$ T cells and NK cells in patients with COVID-19 and immune dysregulation (98). In fact, some pro-inflammatory cytokines, such as IL-6, may block the antiviral immune response by favoring T cells' exhaustion (102). Diao et al. further characterized the exhaustion status of 14 patients with COVID-19. They noted an increasing PD-1 and Tim-3 expression on T cells as patients progressed from prodromal to overtly symptomatic stages (126). Whether this reflects the emergence of exhaustive T cells with a defective capacity to eliminate the virus or a normal evolution of the immune response against the virus remains to be determined. If greater severity of disease is seen in patients with a higher frequency of exhausted T cells, a potential therapeutic approach could be attempted to block those inhibitory receptors, unleashing the T cell response against the virus.

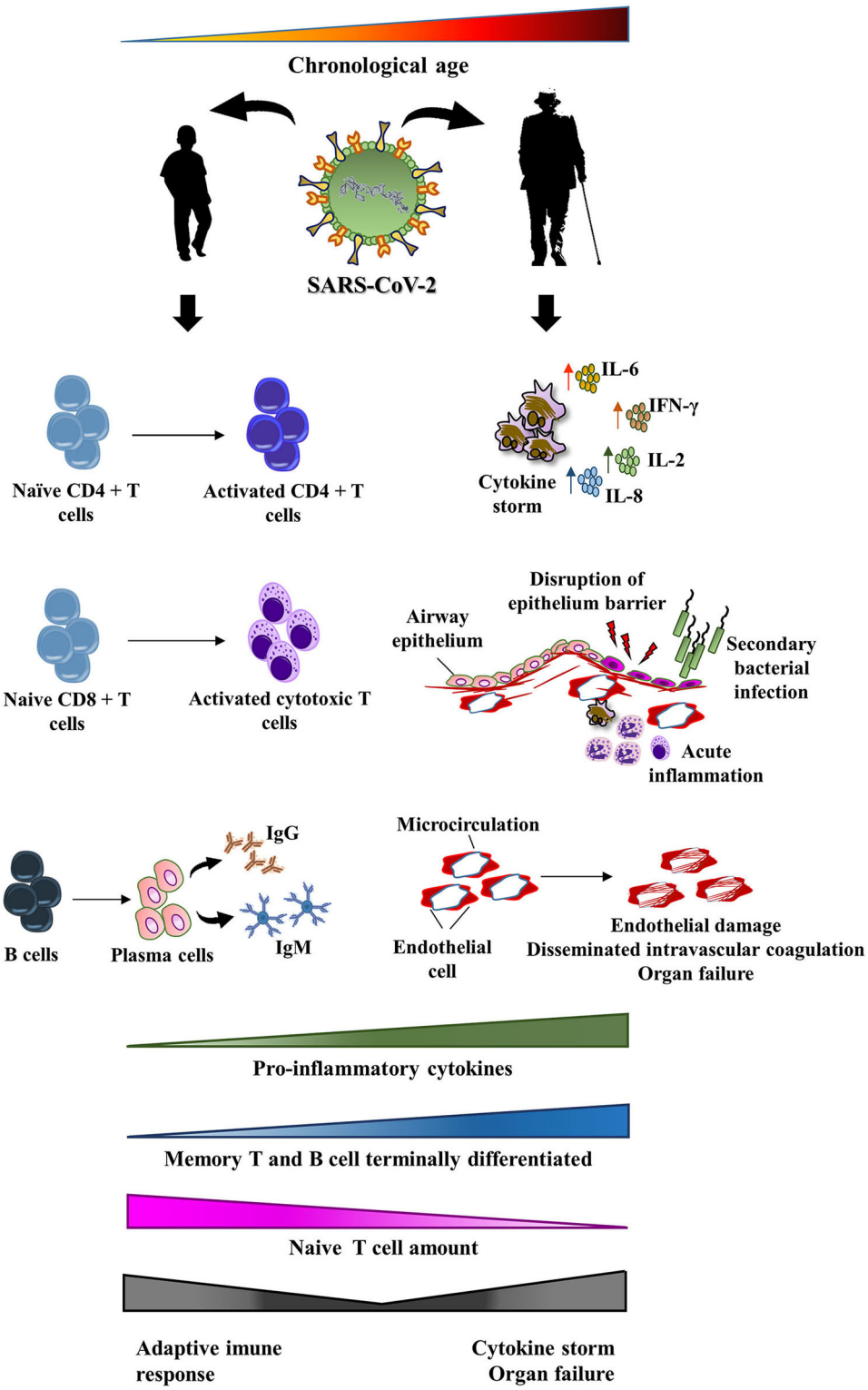


FIGURE 2 | Potential impact of immunosenescence on the pathogenesis of COVID-19. SARS-CoV-2 infection may affect all age ranges, from children to the elderly. Among children, a mild-symptom disease usually occurs. They frequently crush the viral infection through an effective adaptive immune response. However, the remodeling of the immune system that happens with aging may lead to modifications in both adaptive and innate immunity. The final result of these changes may trigger a maladaptive immune response against SARS-CoV-2. In fact, the elderly are an at-risk group to a more aggressive disease that includes cytokine release syndrome, disruption of intrinsic lung defense, secondary bacterial pneumonia, endothelial injury, and end organ damage.

The diminished naïve T cell repository observed among the elderly may dramatically affect the adaptive immune response against SARS-CoV-2, since fewer naïve T cells will be capable of responding to new infections (127, 128). Furthermore, there is also a reduction in the number of regulatory T cells with aging, which help keep the immune system under tighter control (129). Since the elderly frequently present with a remodeled adaptive immune response, they may fail to enhance antibody production. Instead, a pro-inflammatory tone characteristic of inflamm-aging may convert the immune response of patients with COVID-19 in a life-threatening cytokine storm. On the contrary, young patients usually present with an enormous number of naïve T cells that had never encountered a virus. Then, naïve T lymphocytes are rapidly primed and innate immunity does not overwhelm the adaptive immune response. This may explain, at least in part, the favorable prognosis observed among young subjects. **Figure 2** shows the possible relationship between immune response in patients with COVID-19 and the remodeling process that takes place in the immune system with aging.

CONCLUSION AND FUTURE PERSPECTIVES

The immune system faces a complex adaptation over time, culminating in functional and phenotyping alterations. The influence of age-related remodeling of the immune system is clinically observed within elderly features (e.g., frailty syndrome) that can be assessed by lab tests. Despite several promising experimental methods, none are clinically validated so far, but certainly shed some light on the pathophysiology of immunosenescence. Novel mechanisms of inflamm-aging may rise in the near future, leading to new potential therapeutic targets for age-related disorders. Different from the chronological age, the "immune age" obtained by population studies may accurately reflect the molecular and cellular changes that occur over time (130). Immunosenescence may explain the lethality amongst the elderly with COVID-19 with a combination of ineffective T cell response, failed antibody production against SARS-CoV-2, and inflamm-aging that terribly collapses the homeostasis, leading to severe organ dysfunction. The biomarkers that are hallmarks of the remodeled immune response have been raised as new potential targets in patients with COVID-19. More studies are warranted to investigate how to help the elderly to elicit a functional adaptive immune response, as well as to diminish the harmful pro-inflammatory state of the disease.

AUTHOR CONTRIBUTIONS

LC: conception and design, review of the literature, composition of the manuscript and final approval. SP, JA, and PC: design, critical review of the literature, composition of the manuscript, and final approval. LR: conception and design, selection of notable articles for review, critical review of the literature, composition of the manuscript, clinical, and translational orientation and final approval. All authors contributed to the article and approved the submitted version.

REFERENCES

1. NIH. Coronavirus (COVID-19). National Institutes of Health (2020). Available online at: https://www.nih.gov/health-information/coronavirus (accessed April 19, 2020).
2. Verity R, Okell LC, Dorigatti I, Winskill P, Whittaker C, Imai N, et al. Estimates of the severity of coronavirus disease 2019: a model-based analysis. *Lancet Infect Dis.* (2020) S1473–3099:30243–7. doi: 10.1016/S1473-3099(20)30243-7
3. Weng NP. Aging of the immune system: how much can the adaptive immune system adapt? *Immunity.* (2006) 24:495–9. doi: 10.1016/j.immuni.2006.05.001
4. Fulop T, Larbi A, Dupuis G, Le Page A, Frost EH, Cohen AA, et al. Immunosenescence and inflamm-aging as two sides of the same coin: friends or foes? *Front Immunol.* (2017) 8:1960. doi: 10.3389/fimmu.2017.01960
5. Sadighi Akha AA. Aging and the immune system: An overview. *J Immunol Methods.* (2018) 463:21–6. doi: 10.1016/j.jim.2018.08.005
6. Rohrmann S. Epidemiology of Frailty in Older People. *Adv Exp Med Biol.* (2020) 1216:21–7. doi: 10.1007/978-3-030-33330-0_3
7. Kovtonyuk LV, Fritsch K, Feng X, Manz MG, Takizawa H. Inflamm-aging of hematopoiesis, hematopoietic stem cells, and the bone marrow microenvironment. *Front Immunol.* (2016) 7:502. doi: 10.3389/fimmu.2016.00502
8. Lindenauer PK, Lagu T, Shieh MS, Pekow PS, Rothberg MB. Association of diagnostic coding with trends in hospitalizations and mortality of patients with pneumonia, 2003-2009. *JAMA.* (2012) 307:1405–13. doi: 10.1001/jama.2012.384
9. Rebelo M, Pereira B, Lima J, Decq-Mota J, Vieira JD, Costa JN. Predictors of in-hospital mortality in elderly patients with bacteraemia admitted to an Internal Medicine ward. *Int Arch Med.* (2011) 4:33. doi: 10.1186/1755-7682-4-33
10. Hazeldine J, Lord JM. Innate immunesenescence: underlying mechanisms and clinical relevance. *Biogerontology.* (2015) 16:187–201. doi: 10.1007/s10522-014-9514-3
11. Hazeldine J, Lord JM. The impact of ageing on natural killer cell function and potential consequences for health in older adults. *Ageing Res Rev.* (2013) 12:1069–78. doi: 10.1016/j.arr.2013.04.003
12. Aiello A, Farzaneh F, Candore G, Caruso C, Davinelli S, Gambino CM, et al. Immunosenescence and its hallmarks: how to oppose aging strategically? A review of potential options for therapeutic intervention. *Front Immunol.* (2019) 10:2247. doi: 10.3389/fimmu.2019.02247
13. Globerson A, Effros RB. Ageing of lymphocytes and lymphocytes in the aged. *Immunol Today.* (2000) 21:515–21. doi: 10.1016/S0167-5699(00)01714-X
14. Pawelec G. Immunosenescence: impact in the young as well as the old? *Mech Ageing Dev.* (1999) 108:1–7.
15. Byun HO, Lee YK, Kim JM, Yoon G. From cell senescence to age-related diseases: differential mechanisms of action of senescence-associated secretory phenotypes. *BMB Rep.* (2015) 48:549–58. doi: 10.5483/BMBRep.2015.48.10.122
16. Biagi E, Candela M, Fairweather-Tait S, Franceschi C, Brigidi P. Aging of the human metaorganism: the microbial counterpart. *Age (Dordr).* (2012) 34:247–67. doi: 10.1007/s11357-011-9217-5
17. Bauer ME, Fuente Mdl. The role of oxidative and inflammatory stress and persistent viral infections in immunosenescence.

Mech Ageing Dev. (2016) 158:27–37. doi: 10.1016/j.mad.2016.01.001
18. Franceschi C, Garagnani P, Vitale G, Capri M, Salvioli S. Inflammaging and 'Garb-aging'. *Trends Endocrinol Metab.* (2017) 28:199–212. doi: 10.1016/j.tem.2016.09.005
19. Bellon M, Nicot C. Telomere dynamics in immune senescence and exhaustion triggered by chronic viral infection. *Viruses.* (2017) 9:289. doi: 10.3390/v9100289
20. Pita-Lopez ML, Gayoso I, DelaRosa O, Casado JG, Alonso C, Muñoz-Gomariz E, et al. Effect of ageing on CMV-specific CD8 T cells from CMV seropositive healthy donors. *Immun Ageing.* (2009) 6:11. doi: 10.1186/1742-4933-6-11
21. Pawelec G, Gouttefangeas C. T-cell dysregulation caused by chronic antigenic stress: the role of CMV in immunosenescence? *Aging Clin Exp Res.* (2006) 18:171–3. doi: 10.1007/BF03327436
22. Weyh C, Krüger K, Strasser B. Physical activity and diet shape the immune system during aging. *Nutrients.* (2020) 12:622. doi: 10.3390/nu12030622
23. Duggal NA, Niemiro G, Harridge SDR, Simpson RJ, Lord JM. Can physical activity ameliorate immunosenescence and thereby reduce age-related multi-morbidity? *Nat Rev Immunol.* (2019) 19:563–72. doi: 10.1038/s41577-019-0177-9
24. Brubaker AL, Rendon JL, Ramirez L, Choudhry MA, Kovacs EJ. Reduced neutrophil chemotaxis and infiltration contributes to delayed resolution of cutaneous wound infection with advanced age. *J Immunol.* (2013) 190:1746–57. doi: 10.4049/jimmunol.1201213
25. Sagiv A, Biran A, Yon M, Simon J, Lowe SW, Krizhanovsky V. Granule exocytosis mediates immune surveillance of senescent cells. *Oncogene.* (2013) 32:1971–7. doi: 10.1038/onc.2012.206
26. Hazeldine J, Hampson P, Lord JM. Reduced release and binding of perforin at the immunological synapse underlies the age-related decline in natural killer cell cytotoxicity. *Aging Cell.* (2012) 11:751–9. doi: 10.1111/j.1474-9726.2012.00839.x
27. Almeida-Oliveira A, Smith-Carvalho M, Porto LC, Cardoso-Oliveira J, Ribeiro AoS, Falcão RR, et al. Age-related changes in natural killer cell receptors from childhood through old age. *Hum Immunol.* (2011) 72:319–29. doi: 10.1016/j.humimm.2011.01.009
28. Sapey E, Greenwood H, Walton G, Mann E, Love A, Aaronson N, et al. Phosphoinositide 3-kinase inhibition restores neutrophil accuracy in the elderly: toward targeted treatments for immunosenescence. *Blood.* (2014) 123:239–48. doi: 10.1182/blood-2013-08-519520
29. Swift ME, Burns AL, Gray KL, DiPietro LA. Age-related alterations in the inflammatory response to dermal injury. *J Invest Dermatol.* (2001) 117:1027–35. doi: 10.1046/j.0022-202x.2001.01539.x
30. Beerman I, Bhattacharya D, Zandi S, Sigvardsson M, Weissman IL, Bryder D, et al. Functionally distinct hematopoietic stem cells modulate hematopoietic lineage potential during aging by a mechanism of clonal expansion. *Proc Natl Acad Sci U S A.* (2010) 107:5465–70. doi: 10.1073/pnas.1000834107
31. Della Bella S, Bierti L, Presicce P, Arienti R, Valenti M, Saresella M, et al. Peripheral blood dendritic cells and monocytes are differently regulated in the elderly. *Clin Immunol.* (2007) 122:220–8. doi: 10.1016/j.clim.2006.09.012
32. Shaw AC, Joshi S, Greenwood H, Panda A, Lord JM. Aging of the innate immune system. *Curr Opin Immunol.* (2010) 22:507–13. doi: 10.1016/j.coi.2010.05.003
33. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, et al. Neutrophil extracellular traps kill bacteria. *Science.* (2004) 303:1532–5. doi: 10.1126/science.1092385
34. Carmona-Rivera C, Khaznadar SS, Shwin KW, Irizarry-Caro JA, O'Neil LJ, Liu Y, et al. Deficiency of adenosine deaminase 2 triggers adenosine-mediated NETosis and TNF production in patients with DADA2. *Blood.* (2019) 134:395–406. doi: 10.1182/blood.2018892752
35. Hazeldine J, Harris P, Chapple IL, Grant M, Greenwood H, Livesey A, et al. Impaired neutrophil extracellular trap formation: a novel defect in the innate immune system of aged individuals. *Aging Cell.* (2014) 13:690–8. doi: 10.1111/acel.12222
36. Butcher SK, Chahal H, Nayak L, Sinclair A, Henriquez NV, Sapey E, et al. Senescence in innate immune responses: reduced neutrophil phagocytic capacity and CD16 expression in elderly humans. *J Leukoc Biol.* (2001) 70:881–6. doi: 10.1189/jlb.70.6.881
37. Wenisch C, Patruta S, Daxböck F, Krause R, Hörl W. Effect of age on human neutrophil function. *J Leukoc Biol.* (2000) 67:40–5. doi: 10.1002/jlb.67.1.40
38. Lord JM, Butcher S, Killampali V, Lascelles D, Salmon M. Neutrophil ageing and immunesenescence. *Mech Ageing Dev.* (2001) 122:1521–35. doi: 10.1016/S0047-6374(01)00285-8
39. Fulop T, Le Page A, Fortin C, Witkowski JM, Dupuis G, Larbi A. Cellular signaling in the aging immune system. *Curr Opin Immunol.* (2014) 29:105–11. doi: 10.1016/j.coi.2014.05.007
40. Fulop T, Larbi A, Douziech N, Fortin C, Guérard KP, Lesur O, et al. Signal transduction and functional changes in neutrophils with aging. *Aging Cell.* (2004) 3:217–26. doi: 10.1111/j.1474-9728.2004.00110.x
41. Fortin CF, Lesur O, Fulop T. Effects of aging on triggering receptor expressed on myeloid cells (TREM)-1-induced PMN functions. *FEBS Lett.* (2007) 581:1173–8. doi: 10.1016/j.febslet.2007.02.029
42. Guayerbas N, Puerto M, Víctor VM, Miquel J, De la Fuente M. Leukocyte function and life span in a murine model of premature immunosenescence. *Exp Gerontol.* (2002) 37:249–56. doi: 10.1016/S0531-5565(01)00190-5
43. Guayerbas N, Catalán M, Víctor VM, Miquel J, De la Fuente M. Relation of behaviour and macrophage function to life span in a murine model of premature immunosenescence. *Behav Brain Res.* (2002) 134:41–8. doi: 10.1016/S0166-4328(01)00449-1
44. Garrido A, Cruces J, Ceprián N, Vara E, de la Fuente M. Oxidative-inflammatory stress in immune cells from adult mice with premature aging. *Int J Mol Sci.* (2019) 20:769. doi: 10.3390/ijms20030769
45. Garrido A, Cruces J, Ceprián N, Hernández-Sánchez C, De la Fuente M. Premature aging in behavior and immune functions in tyrosine hydroxylase haploinsufficient female mice. A longitudinal study. *Brain Behav Immun.* (2018) 69:440–55. doi: 10.1016/j.bbi.2018.01.003
46. da Costa RM, Rodrigues D, Pereira CA, Silva JF, Alves JV, Lobato NS, et al. Nrf2 as a potential mediator of cardiovascular risk in metabolic diseases. *Front Pharmacol.* (2019) 10:382. doi: 10.3389/fphar.2019.00382
47. Viveros MP, Arranz L, Hernanz A, Miquel J, De la Fuente M. A model of premature aging in mice based on altered stress-related behavioral response and immunosenescence. *Neuroimmunomodulation.* (2007) 14:157–62. doi: 10.1159/000110640
48. Monti D, Ostan R, Borelli V, Castellani G, Franceschi C. Inflammaging and human longevity in the omics era. *Mech Ageing Dev.* (2017) 165:129–38. doi: 10.1016/j.mad.2016.12.008
49. Fülöp T, Dupuis G, Witkowski JM, Larbi A. The role of immunosenescence in the development of age-related diseases. *Rev Invest Clin.* (2016) 68:84–91.
50. Baylis D, Bartlett DB, Syddall HE, Ntani G, Gale CR, Cooper C, et al. Immune-endocrine biomarkers as predictors of frailty and mortality: a 10-year longitudinal study in community-dwelling older people. *Age (Dordr).* (2013) 35:963–71. doi: 10.1007/s11357-012-9396-8
51. Bruunsgaard H, Ladelund S, Pedersen AN, Schroll M, Jørgensen T, Pedersen BK. Predicting death from tumour necrosis factor-alpha and interleukin-6 in 80-year-old people. *Clin Exp Immunol.* (2003) 132:24–31. doi: 10.1046/j.1365-2249.2003.02137.x
52. Harris TB, Ferrucci L, Tracy RP, Corti MC, Wacholder S, Ettinger WH, et al. Associations of elevated interleukin-6 and C-reactive protein levels with mortality in the elderly. *Am J Med.* (1999) 106:506–12. doi: 10.1016/S0002-9343(99)00066-2
53. Pawelec G. Hallmarks of human "immunosenescence": adaptation or dysregulation? *Immun Ageing.* (2012) 9:15. doi: 10.1186/1742-4933-9-15
54. Goronzy JJ, Fang F, Cavanagh MM, Qi Q, Weyand CM. Naive T cell maintenance and function in human aging. *J Immunol.* (2015) 194:4073–80. doi: 10.4049/jimmunol.1500046
55. Di Benedetto S, Derhovanessian E, Steinhagen-Thiessen E, Goldeck D, Müller L, Pawelec G. Impact of age, sex and CMV-infection on peripheral T cell phenotypes: results from the Berlin BASE-II Study. *Biogerontology.* (2015) 16:631–43. doi: 10.1007/s10522-015-9563-2
56. Olsson J, Wikby A, Johansson B, Löfgren S, Nilsson BO, Ferguson FG. Age-related change in peripheral blood T-lymphocyte subpopulations and cytomegalovirus infection in the very old: the Swedish longitudinal OCTO immune study. *Mech Ageing Dev.* (2000) 121:187–201. doi: 10.1016/S0047-6374(00)00210-4
57. Strindhall J, Skog M, Ernerudh J, Bengner M, Löfgren S, Matussek A, et al. The inverted CD4/CD8 ratio and associated parameters in 66-year-old

individuals: the Swedish HEXA immune study. *Age (Dordr).* (2013) 35:985–91. doi: 10.1007/s11357-012-9400-3
58. Plunkett FJ, Franzese O, Finney HM, Fletcher JM, Belaramani LL, Salmon M, et al. The loss of telomerase activity in highly differentiated CD8+CD28–CD27– T cells is associated with decreased Akt (Ser473) phosphorylation. *J Immunol.* (2007) 178:7710–9. doi: 10.4049/jimmunol.178.12.7710
59. Larbi A, Dupuis G, Khalil A, Douziech N, Fortin C, Fülöp T. Differential role of lipid rafts in the functions of CD4+ and CD8+ human T lymphocytes with aging. *Cell Signal.* (2006) 18:1017–30. doi: 10.1016/j.cellsig.2005.08.016
60. Taams LS, Smith J, Rustin MH, Salmon M, Poulter LW, Akbar AN. Human anergic/suppressive CD4(+)CD25(+) T cells: a highly differentiated and apoptosis-prone population. *Eur J Immunol.* (2001) 31:1122–31. doi: 10.1002/1521-4141(200104)31:4<1122::AID-IMMU1122>3.0.CO;2-P
61. Martins PN, Tullius SG, Markmann JF. Immunosenescence and immune response in organ transplantation. *Int Rev Immunol.* (2014) 33:162–73. doi: 10.3109/08830185.2013.829469
62. Seyda M, Quante M, Uehara H, Slegtenhorst BR, Elkhal A, Tullius SG. Immunosenescence in renal transplantation: a changing balance of innate and adaptive immunity. *Curr Opin Organ Transplant.* (2015) 20:417–23. doi: 10.1097/MOT.0000000000000210
63. Haynes L, Lefebvre JS. Age-related deficiencies in antigen-specific CD4 T cell responses: lessons from mouse models. *Aging Dis.* (2011) 2:374–81.
64. Lang A, Nikolich-Zugich J. Functional CD8 T cell memory responding to persistent latent infection is maintained for life. *J Immunol.* (2011) 187:3759–68. doi: 10.4049/jimmunol.1100666
65. Sadighi Akha AA, Miller RA. Signal transduction in the aging immune system. *Curr Opin Immunol.* (2005) 17:486–91. doi: 10.1016/j.coi.2005.07.004
66. Krogsgaard M, Huppa JB, Purbhoo MA, Davis MM. Linking molecular and cellular events in T-cell activation and synapse formation. *Semin Immunol.* (2003) 15:307–15. doi: 10.1016/j.smim.2003.09.002
67. Decman V, Laidlaw BJ, Doering TA, Leng J, Ertl HC, Goldstein DR, et al. Defective CD8 T cell responses in aged mice are due to quantitative and qualitative changes in virus-specific precursors. *J Immunol.* (2012) 188:1933–41. doi: 10.4049/jimmunol.1101098
68. Garcia GG, Miller RA. Age-dependent defects in TCR-triggered cytoskeletal rearrangement in CD4+ T cells. *J Immunol.* (2002) 169:5021–7. doi: 10.4049/jimmunol.169.9.5021
69. Garcia GG, Miller RA. Age-related defects in CD4+ T cell activation reversed by glycoprotein endopeptidase. *Eur J Immunol.* (2003) 33:3464–72. doi: 10.1002/eji.200324310
70. Haynes L, Linton PJ, Eaton SM, Tonkonogy SL, Swain SL. Interleukin 2, but not other common gamma chain-binding cytokines, can reverse the defect in generation of CD4 effector T cells from naive T cells of aged mice. *J Exp Med.* (1999) 190:1013–24. doi: 10.1084/jem.190.7.1013
71. Wherry EJ, Kurachi M. Molecular and cellular insights into T cell exhaustion. *Nat Rev Immunol.* (2015) 15:486–99. doi: 10.1038/nri3862
72. Anderson AC, Joller N, Kuchroo VK. Lag-3, Tim-3, and TIGIT: co-inhibitory receptors with specialized functions in immune regulation. *Immunity.* (2016) 44:989–1004. doi: 10.1016/j.immuni.2016.05.001
73. Parry RV, Chemnitz JM, Frauwirth KA, Lanfranco AR, Braunstein I, Kobayashi SV, et al. CTLA-4 and PD-1 receptors inhibit T-cell activation by distinct mechanisms. *Mol Cell Biol.* (2005) 25:9543–53. doi: 10.1128/MCB.25.21.9543-9553.2005
74. Shimada Y, Hayashi M, Nagasaka Y, Ohno-Iwashita Y, Inomata M. Age-associated up-regulation of a negative co-stimulatory receptor PD-1 in mouse CD4+ T cells. *Exp Gerontol.* (2009) 44:517–22. doi: 10.1016/j.exger.2009.05.003
75. Channappanavar R, Twardy BS, Krishna P, Suvas S. Advancing age leads to predominance of inhibitory receptor expressing CD4 T cells. *Mech Ageing Dev.* (2009) 130:709–12. doi: 10.1016/j.mad.2009.08.006
76. Lee KA, Shin KS, Kim GY, Song YC, Bae EA, Kim IK, et al. Characterization of age-associated exhausted $CD8^+$ T cells defined by increased expression of Tim-3 and PD-1. *Aging Cell.* (2016) 15:291–300. doi: 10.1111/acel.12435
77. Song Y, Wang B, Song R, Hao Y, Wang D, Li Y, et al. T-cell immunoglobulin and ITIM domain contributes to CD8. *Aging Cell.* (2018) 17:e12716. doi: 10.1111/acel.12716
78. Johnston RJ, Comps-Agrar L, Hackney J, Yu X, Huseni M, Yang Y, et al. The immunoreceptor TIGIT regulates antitumor and antiviral CD8(+) T cell effector function. *Cancer Cell.* (2014) 26:923–37. doi: 10.1016/j.ccell.2014.10.018
79. Chew GM, Fujita T, Webb GM, Burwitz BJ, Wu HL, Reed JS, et al. TIGIT marks exhausted T Cells, correlates with disease progression, and serves as a target for immune restoration in HIV and SIV infection. *PLoS Pathog.* (2016) 12:e1005349. doi: 10.1371/journal.ppat.1005349
80. Jiang N, He J, Weinstein JA, Penland L, Sasaki S, He XS, et al. Lineage structure of the human antibody repertoire in response to influenza vaccination. *Sci Transl Med.* (2013) 5:171ra19. doi: 10.1126/scitranslmed.3004794
81. Gibson KL, Wu YC, Barnett Y, Duggan O, Vaughan R, Kondeatis E, et al. B-cell diversity decreases in old age and is correlated with poor health status. *Aging Cell.* (2009) 8:18–25. doi: 10.1111/j.1474-9726.2008.00443.x
82. Frasca D, Blomberg BB. Aging affects human B cell responses. *J Clin Immunol.* (2011) 31:430–5. doi: 10.1007/s10875-010-9501-7
83. Stavnezer J, Guikema JE, Schrader CE. Mechanism and regulation of class switch recombination. *Annu Rev Immunol.* (2008) 26:261–92. doi: 10.1146/annurev.immunol.26.021607.090248
84. Frasca D, Landin AM, Lechner SC, Ryan JG, Schwartz R, Riley RL, et al. Aging down-regulates the transcription factor E2A, activation-induced cytidine deaminase, and Ig class switch in human B cells. *J Immunol.* (2008) 180:5283–90. doi: 10.4049/jimmunol.180.8.5283
85. Frasca D, Diaz A, Romero M, Landin AM, Phillips M, Lechner SC, et al. Intrinsic defects in B cell response to seasonal influenza vaccination in elderly humans. *Vaccine.* (2010) 28:8077–84. doi: 10.1016/j.vaccine.2010.10.023
86. Simell B, Vuorela A, Ekström N, Palmu A, Reunanen A, Meri S, et al. Aging reduces the functionality of anti-pneumococcal antibodies and the killing of Streptococcus pneumoniae by neutrophil phagocytosis. *Vaccine.* (2011) 29:1929–34. doi: 10.1016/j.vaccine.2010.12.121
87. Tortorella C, Piazzolla G, Spaccavento F, Vella F, Pace L, Antonaci S. Regulatory role of extracellular matrix proteins in neutrophil respiratory burst during aging. *Mech Ageing Dev.* (2000) 119:69–82. doi: 10.1016/S0047-6374(00)00171-8
88. Schwarzenbach HR, Nakagawa T, Conroy MC, de Weck AL. Skin reactivity, basophil degranulation and IgE levels in ageing. *Clin Allergy.* (1982) 12:465–73. doi: 10.1111/j.1365-2222.1982.tb01645.x
89. Smith P, Dunne DW, Fallon PG. Defective *in vivo* induction of functional type 2 cytokine responses in aged mice. *Eur J Immunol.* (2001) 31:1495–502. doi: 10.1002/1521-4141(200105)31:5<1495::AID-IMMU1495>3.0.CO;2-8
90. Nel HJ, Hams E, Saunders SP, Mangan NE, Smith P, Atzberger A, et al. Impaired basophil induction leads to an age-dependent innate defect in type 2 immunity during helminth infection in mice. *J Immunol.* (2011) 186:4631–9. doi: 10.4049/jimmunol.1002995
91. Mathur SK, Schwantes EA, Jarjour NN, Busse WW. Age-related changes in eosinophil function in human subjects. *Chest.* (2008) 133:412–9. doi: 10.1378/chest.07-2114
92. Ciaramella A, Spalletta G, Bizzoni F, Salani F, Caltagirone C, Bossù P. Effect of age on surface molecules and cytokine expression in human dendritic cells. *Cell Immunol.* (2011) 269:82–9. doi: 10.1016/j.cellimm.2011.04.010
93. Fujihashi K, Kiyono H. Mucosal immunosenescence: new developments and vaccines to control infectious diseases. *Trends Immunol.* (2009) 30:334–43. doi: 10.1016/j.it.2009.04.004
94. Gupta S. Role of dendritic cells in innate and adaptive immune response in human aging. *Exp Gerontol.* (2014) 54:47–52. doi: 10.1016/j.exger.2013.12.009
95. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet.* (2020) 395:497–506. doi: 10.1016/S0140-6736(20)30183-5
96. Jin Y, Yang H, Ji W, Wu W, Chen S, Zhang W, et al. Virology, epidemiology, pathogenesis, and control of COVID-19. *Viruses.* (2020) 12:372. doi: 10.3390/v12040372
97. Xu Z, Shi L, Wang Y, Zhang J, Huang L, Zhang C, et al. Pathological findings of COVID-19 associated with acute respiratory distress syndrome. *Lancet Respir Med.* (2020) 8:420–2. doi: 10.1016/S2213-2600(20)30076-X
98. Giamarellos-Bourboulis EJ, Netea MG, Rovina N, Akinosoglou K, Antoniadou A, Antonakos N, et al. Complex immune dysregulation in

COVID-19 patients with severe respiratory failure. *Cell Host Microbe.* (2020) 27:992–1000.e3. doi: 10.1016/j.chom.2020.04.009
99. Tai W, He L, Zhang X, Pu J, Voronin D, Jiang S, et al. Characterization of the receptor-binding domain (RBD) of 2019 novel coronavirus: implication for development of RBD protein as a viral attachment inhibitor and vaccine. *Cell Mol Immunol.* (2020) 17:613–20. doi: 10.1038/s41423-020-0400-4
100. Hoffmann M, Kleine-Weber H, Schroeder S, Krüger N, Herrler T, Erichsen S, et al. SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor. *Cell.* (2020) 181:271–80.e8. doi: 10.1016/j.cell.2020.02.052
101. Canan CH, Gokhale NS, Carruthers B, Lafuse WP, Schlesinger LS, Torrelles JB, et al. Characterization of lung inflammation and its impact on macrophage function in aging. *J Leukoc Biol.* (2014) 96:473–80. doi: 10.1189/jlb.4A0214-093RR
102. Velazquez-Salinas L, Verdugo-Rodriguez A, Rodriguez LL, Borca MV. The role of interleukin 6 during viral infections. *Front Microbiol.* (2019) 10:1057. doi: 10.3389/fmicb.2019.01057
103. Xiong Z, Leme AS, Ray P, Shapiro SD, Lee JS. CX3CR1+ lung mononuclear phagocytes spatially confined to the interstitium produce TNF-α and IL-6 and promote cigarette smoke-induced emphysema. *J Immunol.* (2011) 186:3206–14. doi: 10.4049/jimmunol.1003221
104. Lagunas-Rangel FA, Chávez-Valencia V. High IL-6/IFN-γ ratio could be associated with severe disease in COVID-19 patients. *J Med Virol.* (2020). doi: 10.1002/jmv.25900. [Epub ahead of print].
105. Wang F, Hou H, Luo Y, Tang G, Wu S, Huang M, et al. The laboratory tests and host immunity of COVID-19 patients with different severity of illness. *JCI Insight.* (2020) 5:e137799. doi: 10.1172/jci.insight.137799
106. Han H, Ma Q, Li C, Liu R, Zhao L, Wang W, et al. Profiling serum cytokines in COVID-19 patients reveals IL-6 and IL-10 are disease severity predictors. *Emerg Microbes Infect.* (2020) 9:1123–30. doi: 10.1080/22221751.2020.1770129
107. Zhang S, Gan J, Chen BG, Zheng D, Zhang JG, Lin RH, et al. Dynamics of peripheral immune cells and their HLA-G and receptor expressions in a patient suffering from critical COVID-19 pneumonia to convalescence. *Clin Transl Immunology.* (2020) 9:e1128. doi: 10.1002/cti2.1128
108. Chen G, Wu D, Guo W, Cao Y, Huang D, Wang H, et al. Clinical and immunological features of severe and moderate coronavirus disease 2019. *J Clin Invest.* (2020) 130:2620–9. doi: 10.1172/JCI137244
109. Day JW, Fox TA, Halsey R, Carpenter B, Kottaridis PD. IL-1 blockade with anakinra in acute leukaemia patients with severe COVID-19 pneumonia appears safe and may result in clinical improvement. *Br J Haematol.* (2020) 190:e80–3. doi: 10.1111/bjh.16873
110. Pontali E, Volpi S, Antonucci G, Castellaneta M, Buzzi D, Tricerri F, et al. Safety and efficacy of early high-dose IV anakinra in severe COVID-19 lung disease. *J Allergy Clin Immunol.* (2020) 146:213–5. doi: 10.1016/j.jaci.2020.05.002
111. Xu X, Han M, Li T, Sun W, Wang D, Fu B, et al. Effective treatment of severe COVID-19 patients with tocilizumab. *Proc Natl Acad Sci U S A.* (2020) 117:10970. doi: 10.1073/pnas.2005615117
112. Camp JV, Jonsson CB. A role for neutrophils in viral respiratory disease. *Front Immunol.* (2017) 8:550. doi: 10.3389/fimmu.2017.00550
113. Zhang B, Zhou X, Zhu C, Feng F, Qiu Y, Feng J, et al. Immune phenotyping based on neutrophil-to-lymphocyte ratio and IgG predicts disease severity and outcome for patients with COVID-19. *medRxiv.* (2020). doi: 10.1101/2020.03.12.20035048
114. Wen W, Su W, Tang H, Le W, Zhang X, Zheng Y, et al. Immune cell profiling of COVID-19 patients in the recovery stageby single-cell sequencing. *Cell Dis.* (2020) 6:31. doi: 10.1038/s41421-020-0168-9
115. Wilk AJ, Rustagi A, Zhao NQ, Roque J, Martínez-Colón GJ, McKechnie JL, et al. A single-cell atlas of the peripheral immune response in patients with severe COVID-19. *Nat Med.* (2020) 26:1070–76. doi: 10.1101/2020.04.17.20069930
116. Zheng M, Gao Y, Wang G, Song G, Liu S, Sun D, et al. Functional exhaustion of antiviral lymphocytes in COVID-19 patients. *Cell Mol Immunol.* (2020) 17:533–5. doi: 10.1038/s41423-020-0402-2
117. Tam A, Wadsworth S, Dorscheid D, Man SF, Sin DD. The airway epithelium: more than just a structural barrier. *Ther Adv Respir Dis.* (2011) 5:255–73. doi: 10.1177/1753465810396539
118. Whitsett JA, Alenghat T. Respiratory epithelial cells orchestrate pulmonary innate immunity. *Nat Immunol.* (2015) 16:27–35. doi: 10.1038/ni.3045
119. Josset L, Menachery VD, Gralinski LE, Agnihothram S, Sova P, Carter VS, et al. Cell host response to infection with novel human coronavirus EMC predicts potential antivirals and important differences with SARS coronavirus. *mBio.* (2013) 4:e00165–13. doi: 10.1128/mBio.00165-13
120. Brandenberger C, Kling KM, Vital M, Christian M. The role of pulmonary and systemic immunosenescence in acute lung injury. *Aging Dis.* (2018) 9:553–65. doi: 10.14336/AD.2017.0902
121. Kling KM, Lopez-Rodriguez E, Pfarrer C, Mühlfeld C, Brandenberger C. Aging exacerbates acute lung injury-induced changes of the air-blood barrier, lung function, and inflammation in the mouse. *Am J Physiol Lung Cell Mol Physiol.* (2017) 312:L1–L12. doi: 10.1152/ajplung.00347.2016
122. Schouten LR, van Kaam AH, Kohse F, Veltkamp F, Bos LD, de Beer FM, et al. Age-dependent differences in pulmonary host responses in ARDS: a prospective observational cohort study. *Ann Intensive Care.* (2019) 9:55. doi: 10.1186/s13613-019-0529-4
123. Zhou F, Yu T, Du R, Fan G, Liu Y, Liu Z, et al. Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: a retrospective cohort study. *Lancet.* (2020) 395:1054–62. doi: 10.1016/S0140-6736(20)30566-3
124. Thevarajan I, Nguyen THO, Koutsakos M, Druce J, Caly L, van de Sandt CE, et al. Breadth of concomitant immune responses prior to patient recovery: a case report of non-severe COVID-19. *Nat Med.* (2020) 26:453–5. doi: 10.1038/s41591-020-0819-2
125. Liu W, Fontanet A, Zhang PH, Zhan L, Xin ZT, Baril L, et al. Two-year prospective study of the humoral immune response of patients with severe acute respiratory syndrome. *J Infect Dis.* (2006) 193:792–5. doi: 10.1086/500469
126. Diao B, Wang C, Tan Y, Chen X, Liu Y, Ning L, et al. Reduction and functional exhaustion of T cells in patients with coronavirus disease 2019 (COVID-19). *medRxiv.* (2020) 11:827. doi: 10.3389/fimmu.2020.00827
127. Nikolich-Zugich J. Ageing and life-long maintenance of T-cell subsets in the face of latent persistent infections. *Nat Rev Immunol.* (2008) 8:512–22. doi: 10.1038/nri2318
128. Becklund BR, Purton JF, Ramsey C, Favre S, Vogt TK, Martin CE, et al. The aged lymphoid tissue environment fails to support naïve T cell homeostasis. *Sci Rep.* (2016) 6:30842. doi: 10.1038/srep30842
129. Fessler J, Ficjan A, Duftner C, Dejaco C. The impact of aging on regulatory T-cells. *Front Immunol.* (2013) 4:231. doi: 10.3389/fimmu.2013.00231
130. Alpert A, Pickman Y, Leipold M, Rosenberg-Hasson Y, Ji X, Gaujoux R, et al. A clinically meaningful metric of immune age derived from high-dimensional longitudinal monitoring. *Nat Med.* (2019) 25:487–95. doi: 10.1038/s41591-019-0381-y

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Medicine

ORIGINAL RESEARCH
published: 11 August 2020
doi: 10.3389/fmed.2020.00524

Early Epidemiological Features of COVID-19 in Nepal and Public Health Response

Santosh Dhakal[1] *and Surendra Karki*[2*]

[1] *W. Harry Feinstone Department of Molecular Microbiology and Immunology, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, United States,* [2] *Department of Epidemiology and Public Health, Himalayan College of Agricultural Sciences and Technology, Kirtipur, Nepal*

Coronavirus disease 2019 (COVID-19), caused by Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), was first reported in late 2019 from Wuhan, China. Considering COVID-19's alarming levels of spread and severity, the World Health Organization (WHO) declared a global pandemic on March 11, 2020. The first case of COVID-19 in Nepal was reported on January 23, 2020. The Government of Nepal implemented different public health measures to contain COVID-19, including border closures and a countrywide lockdown. We collected the daily data provided by the Ministry of Health and Population (MoHP) of the Government of Nepal and illustrated the early epidemiological characteristics of COVID-19 in Nepal. By May 31, 2020, 1,572 cases and eight deaths were reported in Nepal associated with COVID-19. The estimate of prevalence for COVID-19 among tested populations was 2.25% (95% CI: 2.15–2.37%) and case-fatality rate was 0.5%. The majority of the cases were young males ($n = 1,454$, 92%), with overall average age being 30.5 years (ranging from 2 months to 81 years) and were mostly asymptomatic. There were only five cases from three districts until the end of March, but cases surged from April and spread to 57 out of 77 districts of Nepal by the end of May 2020 despite the continuous lockdown. Most of these cases are from the southern plains of Nepal, bordering India. As the effect of COVID-19 is expected to persist longer, the Government of Nepal should make appropriate strategies for loosening lockdowns in a phase-wise manner while maintaining social distancing and personal hygiene and increasing its testing, tracking, and medical capacity.

Keywords: severe acute respiratory syndrome coronavirus-2, coronavirus disease 2019, epidemiology, public health response, Nepal

OPEN ACCESS

Edited by:
Zisis Kozlakidis, International Agency For Research On Cancer (IARC), France

Reviewed by:
Mohamed Izham Mohamed Ibrahim, Qatar University, Qatar
Meghnath Dhimal, Nepal Health Research Council, Nepal

***Correspondence:**
Surendra Karki
karkisuren@fulbrightmail.org

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: *06 June 2020*
Accepted: *27 July 2020*
Published: *11 August 2020*

Citation:
Dhakal S and Karki S (2020) Early Epidemiological Features of COVID-19 in Nepal and Public Health Response. Front. Med. 7:524. doi: 10.3389/fmed.2020.00524

INTRODUCTION

Coronaviruses (CoVs) are enveloped, positive-sense, single-stranded RNA viruses with a comparatively larger genome size (30 Kb), belonging to the order *Nidovirales,* family *Coronaviridae,* and subfamily *Coronavirinae* (1). The subfamily is further divided into four genera: alpha, beta, gamma, and delta coronaviruses. Those infecting mammals fall within alpha and beta CoVs (2). When contracted by farm animals, CoVs are known to cause severe economic losses for a considerable time. Transmissible Gastroenteritis Virus (TGEV) and Porcine Epidemic Diarrhea Virus (PEDV) in pigs, and Bovine Coronaviruses (BCoVs) in cattle are a few such examples (3, 4).

The PEDV outbreak in the US pig industry in 2013 was characterized by severe gastroenteritis in piglets. This outbreak killed over 7 million pigs within a year, which was 10% of the total pig population in the US (4). CoVs also cause Infectious Bronchitis in poultry, resulting in huge economic losses in the poultry industry. They are also transmissible to dogs and cats. In humans, CoVs (HCoV-NL63, HCoV-229E, HCoV-OC43, and KHU1) were traditionally known to cause mild respiratory infections until the emergence of Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) (5). SARS-CoV emerged from Guangdong Province, China, in November 2002 and spread rapidly to at least 27 countries, leading to over 8,000 reported cases and over 750 deaths, with about a 10% case-fatality rate (6). Within a decade of the SARS-CoV epidemic, another novel coronavirus infection was reported from Saudi Arabia, in June 2012 (6). This virus, later named Middle East Respiratory Syndrome Coronavirus (MERS-CoV), had around a 34% case-fatality rate and resulted in a total of nearly 2,500 laboratory-confirmed cases and over 850 associated deaths from 27 countries as of November 2019 (7). In December 2019, a series of viral pneumonia cases were reported from Wuhan, Hubei Province, China. The causative agent, a novel beta coronavirus, was first named as 2019 novel coronavirus (2019-nCoV) (8). 2019-nCoV was ultimately renamed as Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) and the disease, characterized by symptoms including fever, shortness of breath, cough, fatigue, and pneumonia, was named Coronavirus Disease 2019 (COVID-19) by the World Health Organization (WHO) (9).

On 30th January 2020, the WHO declared that the COVID-19 outbreak constituted a Public Health Emergency of International Concern (PHEIC), which was eventually declared a global pandemic on 11th March 2020, owing to the alarming levels of spread and severity of COVID-19 (10). It is most likely that, similar to SARS and MERS coronaviruses, SARS-CoV-2 also originated from bat reservoirs. Studies have shown around an 80% genetic sequence homology between SARS-CoV-2 and SARS-CoV, while the resemblance of SARS-CoV-2 with bat coronaviruses is over 95% (11, 12). Bat coronaviruses require intermediate animal hosts before the spillover occurs in humans. For SARS-CoV and MERS-CoV, palm civet and dromedary camels, respectively, were found to serve as intermediate hosts (6). Pangolin coronaviruses had over a 90% similarity to SARS-CoV-2 but evidence contrasts regarding the possibility of pangolins being the intermediate host (13, 14). SARS-CoV-2 can infect animals including ferrets, domestic cats, tigers, and rhesus macaques either naturally or experimentally, but the actual intermediate host which contributed in virus transmission dynamics is not known yet (15–17).

As per the WHO's situation report from 31st May 2020, more than 5.9 million cases of COVID-19 were reported globally, with over 365,000 deaths (18). The US alone has reported more than 1.7 million cases and over 100,000 deaths (19). Other countries most severely affected with higher number of cases of COVID-19 include Brazil, Russia, Spain, the UK, India, Italy, Peru, Germany, and Turkey (9). Early epidemiological studies from China and the US indicated that older age and patients with underlying health conditions were at greater risk of hospitalization, intensive care unit (ICU) admission, and death due to COVID-19 (20, 21). A recent retrospective study from New York also showed that older age and chronic pulmonary and cardiac diseases were independently associated with in-hospital mortality with COVID-19 (22).

As of May 31, 2020, COVID-19 has been reported from 10 of 11 member countries in the WHO South-East Asia region. The highest number of cases have been reported from India, Bangladesh, and Indonesia (23). In Nepal, the first case of COVID-19 was officially reported on 23rd January 2020 in a 32-year-old man who returned from Wuhan, China (24). The second case was detected after two months on 23rd March. By May 31, 1,572 cases and eight deaths were reported from Nepal (25). The increasing situation of COVID-19 will be challenging for countries like Nepal where the health infrastructure is fragile and less equipped. In Nepal, there are only 194 hospitals with ICU facilities, with a capacity of 26,930 hospital beds, 3,076 isolation beds, 1,595 ICU beds, and 840 ventilators. In total, 111 hospitals run COVID-19 clinics while 13 hospitals are designated as level-I COVID-19 hospitals, 12 hospitals as level-II COVID-19 hospitals, and three hospitals as level-III COVID hospitals (26). In this article, we describe the early epidemiological features of COVID-19 in Nepal, its spatiotemporal distribution, the public health response taken by the Government of Nepal, and the way forward.

METHODS

Study Design

This is a descriptive epidemiological study to highlight the early epidemiological features of COVID-19 cases in Nepal.

Study Area

Nepal is a landlocked country surrounded by India in the south, east, and west and China in the north. Nepal has a population of around 30 million (27). Politically, Nepal is divided into seven provinces, 77 districts, and 753 local bodies. Geographically, it is divided into Terai (southern plains bordering to India), hills, and mountains (Himalayan range).

Data Collection

The COVID-19 cases and Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) testing data for this study were compiled using the publicly available official situation reports of the Ministry of Health and Population (MoHP) of the Government of Nepal (25). The MoHP made these data public through daily press meets and through national television broadcasts and MoHP's social media page. The daily situation reports are available from MoHP's website: https://drive.google.com/drive/folders/1QhLMbT76t6Zu1sFy5qlB5aoDbHVAcnHx. This study includes data from January 23, 2020 to May 31, 2020 to understand the early epidemiological features of COVID-19 cases in Nepal. COVID-19 cases in Nepal, as defined by the MoHP, included any individual who had RT-PCR tested positive for SARS-CoV-2 virus infection.

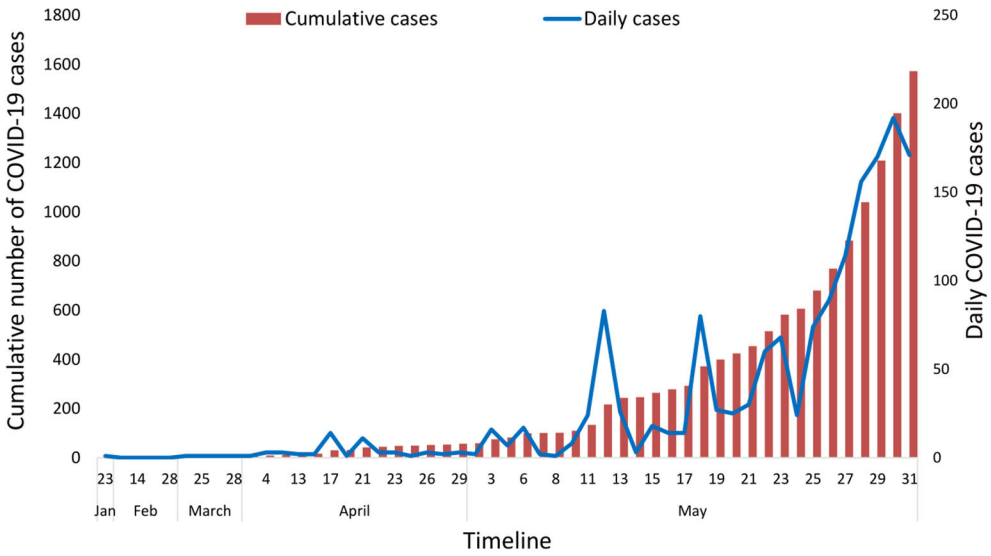


FIGURE 1 | Epidemic curve of COVID-19 cases in Nepal.

Statistical Analysis

The daily data were collated in Microsoft Excel 2016. The graphs were created using the same version of Microsoft Excel. Descriptive statistics of the age distribution of confirmed COVID-19 cases such as the mean, median, minimum, maximum, standard deviation, and quartiles were calculated using the Epi Info version 7.2.3.1 developed by the Center for Disease Control and Prevention (CDC) of the United States (https://www.cdc.gov/epiinfo/index.html).

The choropleth maps, which helps to show the spatial patterns by shading the geographical areas in different colors, were created using the open-access software, QGIS version 3.10.3 (https://www.qgis.org/en/site/) to show the spatial distribution of COVID-19 cases in Nepal in three time periods (January-March; April and May 2020). We aggregated January-March as there were only a few cases (five) in total by the end of March.

RESULTS

The number of COVID-19 confirmed cases in Nepal reached 1,572 by May 31, 2020, after it was first confirmed in the country on January 23, 2020. The first case included a student who had returned from Wuhan, China, who, being aware of the coronavirus outbreak, visited the hospital in Kathmandu for a medical check-up (28). Among the total infected, 220 individuals were discharged from the hospital after testing negative. The epidemic curve based on daily data showed that cases have started rapidly rising since May 2020 (**Figure 1**). During this same period, 69,587 samples were tested using RT-PCR in 20 laboratories distributed across the country, with the majority of the tests being conducted at the National Public Health Laboratory (NPHL) based in the capital city, Kathmandu (**Figure 2**). This early epidemiological data indicates that the prevalence of COVID-19 among the tested individuals in Nepal was 2.25% (95% CI: 2.15–2.37%) (n = 1,572/69,587). More than 95% of these cases were asymptomatic. The tested individuals were mostly people who came in contact with the confirmed cases identified through contact tracing or those in quarantine set up by the government who had returned from foreign countries, the majority of whom returned from India. The earlier cases in Nepal up to mid-April 2020 had a travel history from countries such as China, France, Qatar, Belgium, the United Arab Emirates, the United Kingdom, and Saudi Arabia. All cases after mid-April were either linked to people coming from India via land or people contracting the virus locally, as all international flights were closed effective from March 23, 2020.

Age and Gender Distribution

Among the 1,572 confirmed cases, eight people (0.5%) had died from COVID-19 in Nepal by May 31, 2020. The first COVID-19 death was reported on May 16, 2020, in a new mother who gave birth to her child on May 6th, 2020 in a hospital in Kathmandu and was discharged. She went to her home in Sidhupalchok district, around 4-h bus travel from Kathmandu, and later developed signs of fever and respiratory difficulties and ultimately died on May 16, 2020. The majority of the other deaths were in quarantine and confirmed as COVID-19 after their deaths.

The majority of the cases confirmed in Nepal were young males (**Figure 3**). 92% (n = 1,454/1,572) of the total cases were males and only 8% (n = 118/1,572) were females (**Figure 3**). This is not surprising as this population was tested most given their higher proportion in quarantine. The average age among the overall cases was 30.5 years (Range: 2 months to 81 years). Disaggregation by gender showed that the average age among the males was 30.4 years (Range: 2 months to 74 years) while the average age among the females was 30.8 years (Range: 4 months to 81 years), showing no statistical difference between the average

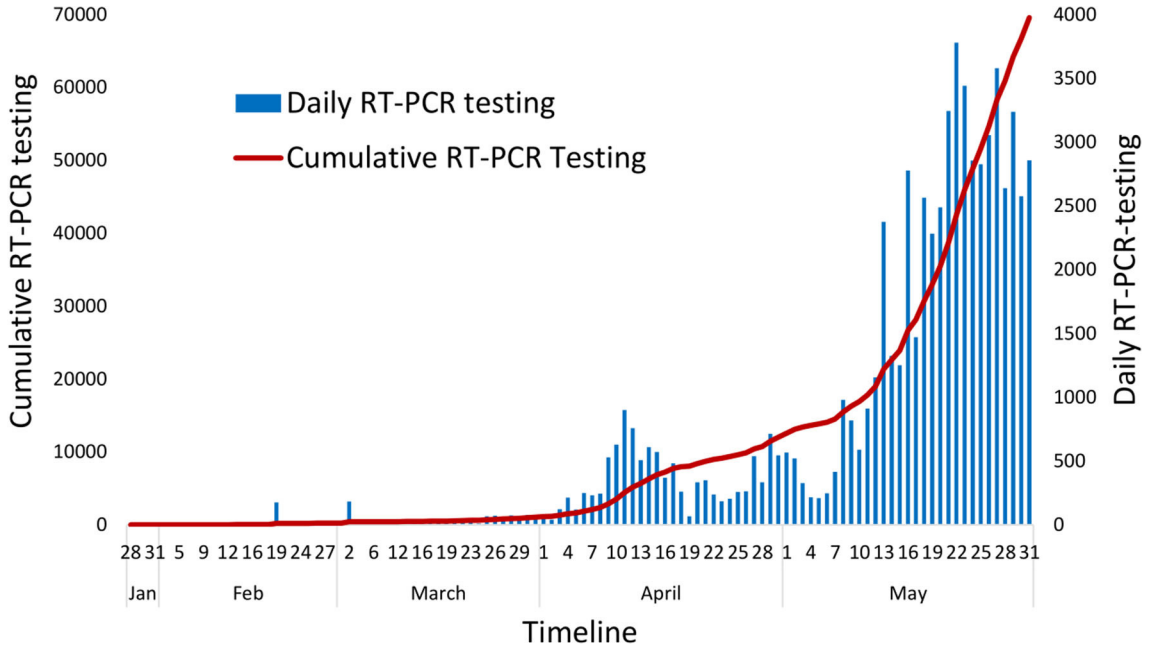


FIGURE 2 | Cumulative and daily RT-PCR testing for COVID-19 diagnosis in Nepal.

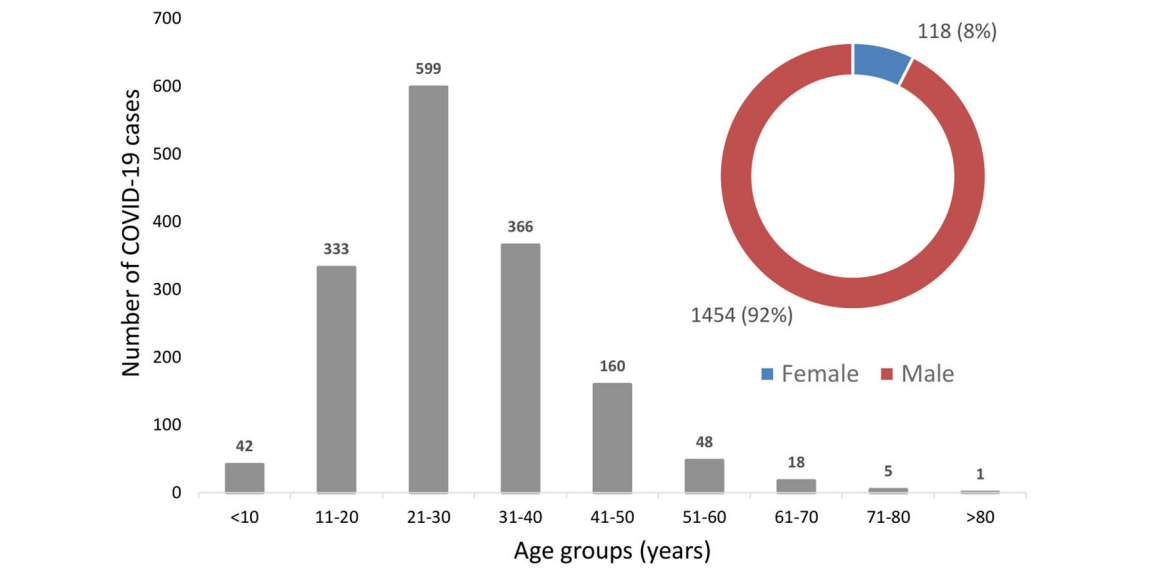


FIGURE 3 | Age and gender-wise distribution of COVID-19 cases in Nepal.

ages by gender ($p = 0.82$). The detailed descriptive statistics of overall age distribution and gender are shown in **Table 1**.

Spatial Patterns

The spatial pattern of COVID-19 cases in Nepal showed that, up to the end of March 2020, cases were reported only from three districts—Kathmandu, Baglung, and Kailali—out of 77 districts of Nepal. The number of districts affected increased to 12 by the end of April 2020 (**Figure 4**). Most of these districts had sporadic cases, except for Udayapur district in the eastern part of Nepal, where a cluster of cases ($n = 28$) was reported from one small village (**Figure 4**). The number of districts affected substantially increased and reached 57 out of 77 districts by the end of May 2020 (**Figure 4**). The majority of the cases were observed in the southern plains of Nepal bordering India in Provinces 1, 2, and 5. Five districts, namely Jhapa, Parsa, Rautahat, Banke, and Kapilvastu reported more than 100 confirmed cases (**Figure 4**). The province-wise distribution shows that Province 2 had the

TABLE 1 | Age distribution of COVID-19 cases in Nepal*.

COVID-19 cases	Mean (Years)	Std. Dev. (Years)	Median (Years)	1st quartile (25%)	3rd quartile (75%)
All ($N = 717$)	30.5	13.4	29	21	37
Male ($N = 616$)	30.4	12.3	29	22	36.5
Female ($N = 101$)	30.8	18.7	28	18	43

Out of 1,572 cases by May 31, 2020, exact age of 717 cases were made public while age of remaining cases were provided in ranges.

highest number of confirmed cases ($n = 624$ out of 1,572), followed by Province 5 ($n = 565$ out of 1,572), Province 1 ($n = 165$ out of 1,572), Karnali province ($n = 123$ out of 1,572), Bagmati province ($n = 45$ out of 1,572), Sudur Pashchim province ($n = 27$ out of 1,572), and Gandaki province ($n = 23$ out of 1,572).

Public Health Measures Adopted by the Government

The Government of Nepal closed all international flights and its international borders on March 23, 2020, after the second COVID-19 case was recorded in Nepal. A day after this, Nepal enforced a nation-wide lockdown on March 24, 2020, which has been extended continuously and has been in effect up to June 14, 2020. The total length of continuous lockdown shall reach 83 days by June 14, 2020. The lockdown modality after June 14, 2020, is not clear at the moment of drafting this manuscript. The government has been using both RT-PCR and antibody-based rapid diagnostic tests (RDT) in parallel to diagnose or screen probable patients. However, only RT-PCR has been considered the confirmatory test. The government increased the number of RT-PCR testing laboratories from one to 20, including four veterinary laboratories. By May 31, 2020, the government had used 111,109 RDT tests to screen people in quarantine or other suspected areas. It has been a challenge for all three tiers of the Government, Federal, Provincial, and Local, to manage the large influx of Nepalese people wanting to return home from India. A small subset of people who have already entered Nepal has been kept in quarantine, which is often reported to be poorly managed due to limited resources. Up till now, the government has been isolating all COVID-19 cases in designated COVID-19 hospitals, irrespective of their clinical situation. This has overwhelmed hospitals with patients who do not need immediate medical attention.

DISCUSSION

This study was conducted to describe the spatiotemporal patterns and early epidemiological features of COVID-19 cases in Nepal from January 23 to May 31, 2020. The findings show that the vast majority of the cases in Nepal were young males and the case fatality rate was 0.5%. The disease was rapidly spreading and reached 57 out of 77 districts from all seven provinces by the end of May 2020.

The strict lockdown, meticulous testing and tracking, and massive isolation of people helped China to reduce the effects of the COVID-19 pandemic (29). Precise and widespread contact tracing and testing, including of asymptomatic individuals, together with social distancing led Taiwan to control COVID-19 in a fascinating way (30, 31). Similar intensive measures were also successfully used by South Korea to reduce COVID-19-associated casualties (32). Likewise, Vietnam, a country of 97 million people with limited resources, has been successful in limiting the spread of COVID-19 through a strong response system, including quick strategic testing and aggressive contact tracing (33). Nepal closed its international borders and enforced a country-wide lockdown early on, when only two cases were identified. The non-pharmaceutical interventions, including border control, lockdown, social distancing, and personal hygiene, helped Nepal in preventing the spread of SARS-CoV-2 during the initial days. However, later on, the effectiveness of the countrywide lockdown has not been observed, as the number of cases surged from 57 cases up to April to 1,572 by the end of May 2020 (25). One major contributor to this surge has been the return of daily wage migrant workers from India (34), where the cases of COVID-19 has been rapidly increasing since April 2020 (10). As Nepal shares its open border with India, citizens desperate to return home found different ways to return to Nepal, including swimming across the Mahakali river bordering two countries (35). There was also significant in-country movement of people wanting to return to their hometown as their livelihood sources in cities were compromised due to the lockdown.

Based on the available data, we estimated the COVID-19 prevalence in Nepal to be 2.25% (n =1,572/69,587). However, it may not represent the actual COVID-19 prevalence because samples from COVID-19 positive individuals are tested at least twice before declaring them COVID-19 negative and added in total numbers, without separating them. This prevalence also might not represent national level prevalence as samples from random populations have not been tested. Early studies reported that COVID-19 patients in Nepal showed few or no symptoms at all (24, 36). The situation updates of the Ministry of Health and Population (MoHP) also indicates that most of the confirmed cases are found through active surveillance and contact tracing rather than patients visiting hospitals with symptoms (25). This is in contrary to what is observed in other countries. The reported death rate (0.5%, n =8/1,572) also appears lower in comparison to the case-fatality rates reported from other countries. As per the mortality analysis carried out by Johns Hopkins University, among the 20 countries most severely affected with COVID-19 as of June 5, 2020, the case-fatality rate is highest in France (15.3%) and lowest in Chile (1.1%) (37). Nepal's neighboring countries, including China, India, Pakistan, and Bangladesh, have 5.5, 2.8, 2.1, and 1.4% case-fatality rates, respectively (37). Though the case fatality rates seem lower, it should not contribute to the relaxing of ongoing pandemic mitigation efforts by the Government of Nepal. The complete genome sequencing of the first SARS-CoV-2, isolated from Nepal, showed more than a 99% sequence homology with viruses isolated from Wuhan, China (38). Further studies are necessary to determine the origin and nature of SARS-CoV-2 circulating in Nepal. Importantly, the

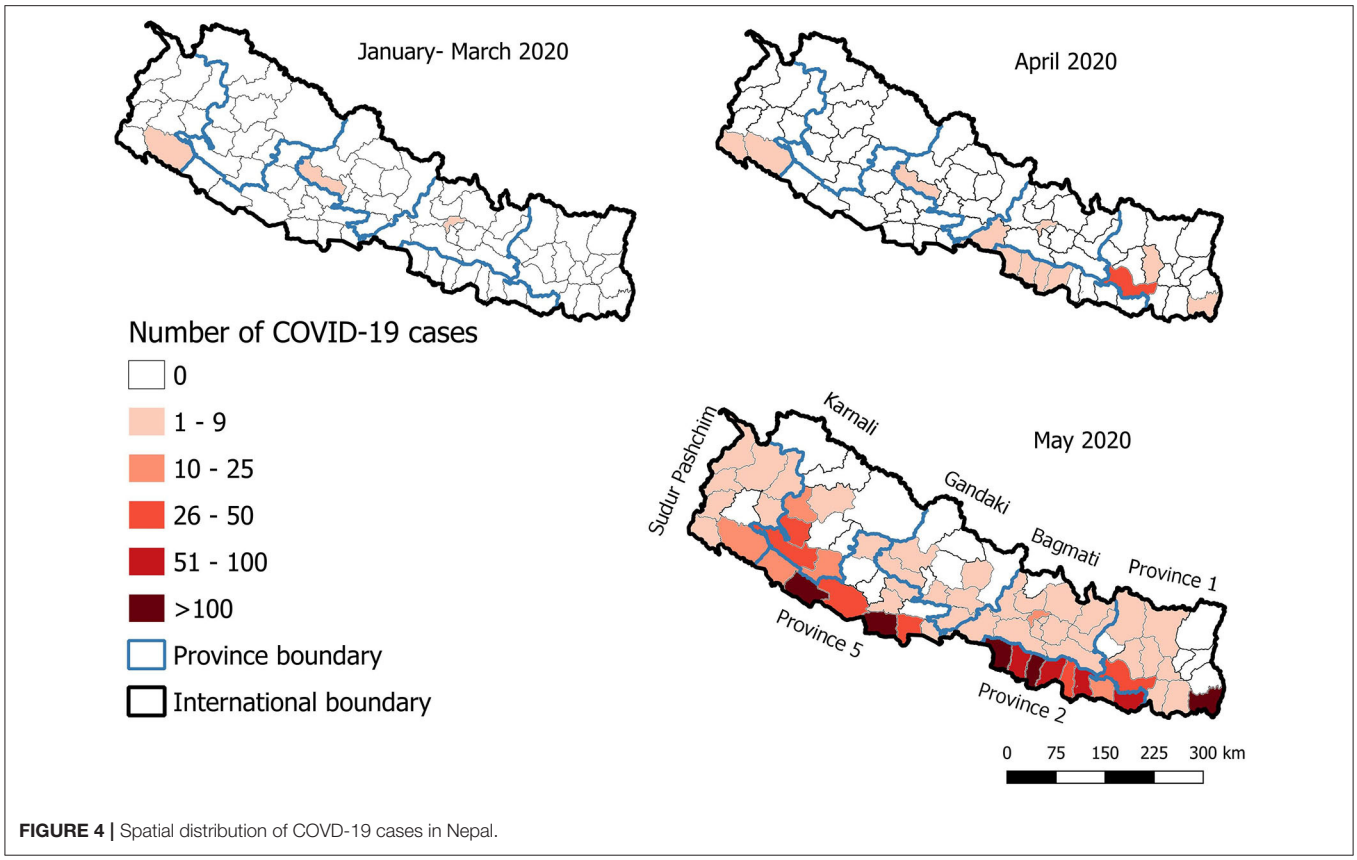


FIGURE 4 | Spatial distribution of COVD-19 cases in Nepal.

true burden of COVID-19 in South Asia, including Nepal, is difficult to estimate due to the low amount of testing and poor documentation (39). Moreover, as of May 31, 2020, the WHO classified the transmission pattern in Nepal as sporadic (18), which means Nepal has not yet observed the larger outbreaks of community-level transmission or the peak of the disease, which might be on its way. There are early signs of it as the WHO Nepal office has indicated that there is some evidence of secondary community transmission and a cluster of cases have been observed in four out of seven provinces of Nepal (40).

Nepal represents a real scenario of low- and middle-income countries (LMICs) where pandemic mitigation efforts are impacted largely by the lack of medical supplies and infrastructure. This includes personal protective equipment (PPE) and ventilators, the limitation of well-trained manpower, the unavailability of enough diagnostic kits; a lack of a proper coordination mechanisms among stakeholders, and poor reporting and documentation of cases (41–44). This pandemic has taught Nepal that it should invest more in research and development in the public health sector, besides the current primary focus on curative medicine. Current use of the laboratory facilities developed by the veterinary sector, to tackle with periodic disease outbreaks in animals including avian influenza viruses (45), for COVID-19 diagnostic purposes further highlights the necessity of intersectoral collaboration in pandemic mitigation efforts. A multisectoral and collaborative one-health approach including animal health, human health, and environmental health professionals (46) will not only be effective in managing the ongoing COVID-19 pandemic control but also will allow for better preparedness against future outbreaks and other imminent problems, such as antimicrobial resistance in Nepal.

COVID-19 has geographically expanded and affected all age groups in Nepal. As of June 6, 2020, the total number of cases and deaths have reached 3,235 and 13, respectively, from 69 out of 77 districts of Nepal (25). The Government of Nepal has been using lockdown as one of its major weapons against COVID-19. If enforced correctly, lockdown measures can effectively reduce the spread of the virus (47). However, the enforcement of a lockdown will likely be less effective if it is continued for long periods of time. Besides this tactic, the government should also consider and be prepared for managing the socio-cultural, economical, and psychological burdens of the lockdown, if it will be continued further. It will be very challenging for countries like Nepal to opt for indefinite lockdown measures given their limited resources and vulnerable socio-economic status.

Strength and Limitations

The strength of this study is that it uses the daily data made public by the MoHP and provides early epidemiological features of COVID-19 cases in Nepal. This study will provide a baseline to compare the epidemiological features of COVID-19 cases in Nepal in the future, as the pattern might change with progression in infection. As only RT-PCR confirmed cases were

included in the study, the data is reliable and provides useful information regarding the spatiotemporal patterns of COVID-19 cases in Nepal. However, this study has some limitations, such as the prevalence calculated in this study perhaps being an underestimation as the number of individuals tested is lower than the total samples tested. In addition, the estimated prevalence is only a reflection of those who are tested rather than the true prevalence at the population level.

CONCLUSION AND RECOMMENDATION

This study provides an overview of the spatiotemporal patterns and early epidemiological features of COVID-19 cases in Nepal. There were 1,572 cases and eight deaths associated with COVID-19 in Nepal by the end of May 2020. The estimate of prevalence for COVID-19 among the tested population was 2.25% and case-fatality rate was 0.5%. The majority of the cases were young and were mostly asymptomatic. The disease had spread to 57 out of 77 districts of Nepal by the end of May 2020, despite the continuous lockdown.

Moving forward, it would be better to identify high-, medium-, and low-risk areas and make appropriate plans for loosening lockdowns in a phase-wise manner to return toward the state of "new normal." As the effect of COVID-19 is likely to persist longer (48), practice of social distancing and good personal hygiene, including the use of face masks, continuous scrutiny at the porous Indian border, increased testing, tracking, and medical capacity, and proper quarantine of cases and high-risk groups should continue in Nepal.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

AUTHOR CONTRIBUTIONS

SD and SK conceived the idea and designed the study and prepared the first draft and revised it. SK collected and analyzed the data. All authors have approved the final version.

ACKNOWLEDGMENTS

We acknowledge the Ministry of Health and Population of the Government of Nepal for making daily data on COVID-19 publicly available.

REFERENCES

1. Fehr AR, Perlman S. Coronaviruses: an overview of their replication and pathogenesis. *Methods Mol Biol.* (2015) 1282:1–23. doi: 10.1007/978-1-4939-2438-7_1
2. Cui J, Li F, Shi ZL. Origin and evolution of pathogenic coronaviruses. *Nat Rev Microbiol.* (2019) 17:181–92. doi: 10.1038/s41579-018-0118-9
3. Saif LJ. Bovine respiratory coronavirus. *Vet Clin North Am Food Anim Pract.* (2010) 26:349–64. doi: 10.1016/j.cvfa.2010.04.005
4. Lin CM, Saif LJ, Marthaler D, Wang Q. Evolution, antigenicity and pathogenicity of global porcine epidemic diarrhea virus strains. *Virus Res.* (2016) 226:20–39. doi: 10.1016/j.virusres.2016.05.023
5. Su S, Wong G, Shi W, Liu J, Lai ACK, Zhou J, et al. Epidemiology, genetic recombination, and pathogenesis of coronaviruses. *Trends Microbiol.* (2016) 24:490–502. doi: 10.1016/j.tim.2016.03.003
6. De Wit E, Van Doremalen N, Falzarano D, Munster VJ. SARS and MERS: recent insights into emerging coronaviruses. *Nat Rev Microbiol.* (2016) 14:523–34. doi: 10.1038/nrmicro.2016.81
7. WHO. *Middle East Respiratory Syndrome Coronavirus (MERS-CoV).* (2019). Available online at: https://www.who.int/emergencies/mers-cov/en/ (accessed May 29, 2020).
8. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet.* (2020) 395:497–506. doi: 10.1016/S0140-6736(20)30183-5
9. WHO. *World Health Organization, Timeline - COVID-19.* (2020). Available online at: https://www.who.int/news-room/detail/27-04-2020-who-timeline---covid-19 (accessed May 29, 2020).
10. WHO. *India Situation Report-9 and 13.* (2020). Available online at: https://www.who.int/india/emergencies/coronavirus-disease-(covid-19)/india-situation-report (accessed July 19, 2020).
11. Lau SKP, Luk HKH, Wong ACP, Li KSM, Zhu L, He Z, et al. Possible bat origin of severe acute respiratory syndrome coronavirus 2. *Emerg Infect Dis.* (2020) 26:1542–7. doi: 10.3201/eid2607.200092
12. Zhou P, Yang XL, Wang XG, Hu B, Zhang L, Zhang W, et al. A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature.* (2020) 579:270–3. doi: 10.1038/s41586-020-2012-7
13. Liu P, Jiang JZ, Wan XF, Hua Y, Li L, Zhou J, et al. Are pangolins the intermediate host of the 2019 novel coronavirus (SARS-CoV-2)? *PLoS Pathog.* (2020) 16:e1008421. doi: 10.1371/journal.ppat.1008421
14. Zhang T, Wu Q, Zhang Z. Probable pangolin origin of SARS-CoV-2 associated with the COVID-19 outbreak. *Curr Biol.* (2020) 30:1578. doi: 10.1016/j.cub.2020.03.063
15. Chandrashekar A, Liu J, Martinot AJ, Mcmahan K, Mercado NB, Peter L, et al. SARS-CoV-2 infection protects against rechallenge in rhesus macaques. *Science.* (2020). doi: 10.1126/science.abc4776. [Epub ahead of print].
16. Shi J, Wen Z, Zhong G, Yang H, Wang C, Huang B, et al. Susceptibility of ferrets, cats, dogs, and other domesticated animals to SARS-coronavirus 2. *Science.* (2020) 368:1016–20. doi: 10.1126/science.abb7015
17. USDA. *USDA Statement on the Confirmation of COVID-19 in a Tiger in New* York (2020).
18. WHO. *Coronavirus Disease (COVID-2019) Situation Reports - 132.* Geneva: World Health Organization (2020).
19. CDC. *Coronavirus Disease 2019 (COVID-19), Cases in the US.* (2020). Available online at: https://www.cdc.gov/coronavirus/2019-ncov/cases-updates/cases-in-us.html (accessed May 29, 2020).
20. Team CC-R. Severe outcomes among patients with coronavirus disease 2019 (COVID-19) - United States, February 12-March 16, 2020. *MMWR Morb Mortal Wkly Rep.* (2020) 69:343–6. doi: 10.15585/mmwr.mm6912e2
21. Team NCPERE. The epidemiological characteristics of an outbreak of 2019 novel coronavirus diseases (COVID-19) in China. *Zhonghua Liu Xing Bing Xue Za Zhi.* (2020) 41:145–51. doi: 10.3760/cma.j.issn.0254-6450.2020.02.003
22. Cummings MJ, Baldwin MR, Abrams D, Jacobson SD, Meyer BJ, Balough EM, et al. Epidemiology, clinical course, and outcomes of critically ill adults with COVID-19 in New York City: a prospective cohort study. *Lancet.* (2020) 395:1763–70. doi: 10.1016/S0140-6736(20)31189-2
23. WHO. *COVID-19 Situation in the WHO South-East Asia Region.* (2020). Available online at: https://www.who.int/southeastasia/outbreaks-and-emergencies/novel-coronavirus-2019 (accessed May 29, 2020).
24. Pun SB, Mandal S, Bhandari L, Jha S, Rajbhandari S, Mishra AK, et al. Understanding COVID-19 in Nepal. *J Nepal Health Res Counc.* (2020) 18:126–7. doi: 10.33314/jnhrc.v18i1.2629

25. MoHP. *COVID-19 Situtation Reports of Ministry of Health and Population, Government of Nepal*. (2020). Available online at: https://covid19.mohp.gov.np (accessed June 5, 2020).
26. MoHP. *Health Sector Emergency Response Plan COVID-19*. (2020). Available online at: https://www.who.int/docs/default-source/nepal-documents/novel-coronavirus/health-sector-emergency-response-plan-covid-19-endorsed-may-2020.pdf?sfvrsn=ef831f44_2 (accessed June 5, 2020).
27. CBS. *National Population and Housing Census 2011 (Population Projection 2011 - 2031)*. Kathmandu: Central Bureau of Statistics, Government of Nepal (2014).
28. Piryani RM, Piryani S, Shah JN. Nepal's response to contain COVID-19 infection. *J Nepal Health Res Counc*. (2020) 18:128–34. doi: 10.33314/jnhrc.v18i1.2608
29. Azman AS, Luquero FJ. From China: hope and lessons for COVID-19 control. *Lancet Infect Dis*. (2020) 20:756–7. doi: 10.1016/S1473-3099(20)30264-4
30. Cheng HY, Jian SW, Liu DP, Ng TC, Huang WT, Lin HH, et al. Contact tracing assessment of COVID-19 transmission dynamics in Taiwan and risk at different exposure periods before and after symptom onset. *JAMA Intern Med*. (2020). doi: 10.1001/jamainternmed.2020.2020. [Epub ahead of print].
31. Steinbrook R. Contact tracing, testing, and control of COVID-19-learning from Taiwan. *JAMA Intern Med*. (2020). doi: 10.1001/jamainternmed.2020.2072. [Epub ahead of print].
32. Choi JY. Covid-19 in South Korea. *Postgrad Med J*. (2020). 96:399–402 doi: 10.1136/postgradmedj-2020-137738
33. Trevisan M, Le LC, Le AV. The COVID-19 pandemic: a view from Vietnam. *Am J Public Health*. (2020) 110:1152–3. doi: 10.2105/AJPH.2020.305751
34. Chalise HN. COVID-19 situation and challenges for Nepal. *Asia Pac J Public Health*. (2020). doi: 10.1177/1010539520932709. [Epub ahead of print].
35. Badu M. *Nepalis are Swimming Across the Mahakali to Get Home*. Kathmandu: The Kathmandu Post (2020).
36. Joshi J, Mishra P, Kamar SB, Sharma ND, Parajuli J, Sharma S, et al. Clinical profile of cases of COVID-19 in Far Western Province of Nepal. *J Nepal Health Res Counc*. (2020) 18:135–7. doi: 10.33314/jnhrc.v18i1.2602
37. Jhu. *How Does Mortality Differ Across Countries?*. Johns Hopkins University, Coronavirus Resource Center (2020). Available online at: https://coronavirus.jhu.edu/data/mortality (accessed June 5, 2020).
38. Sah R, Rodriguez-Morales AJ, Jha R, Chu DKW, Gu H, Peiris M, et al. Complete genome sequence of a 2019 novel coronavirus (SARS-CoV-2) strain isolated in Nepal. *Microbiol Resour Announc*. (2020) 9:e00169–20. doi: 10.1128/MRA.00169-20
39. Bhutta ZA, Basnyat B, Saha S, Laxminarayan R. Covid-19 risks and response in South Asia. *BMJ*. (2020) 368:m1190. doi: 10.1136/bmj.m1190
40. WHO. *Situtation Update #7 - Coronavirus Disease 2019 (COVID-19)*. Kathmandu: WHO Country Office for Nepal (2020).
41. Asim M, Sathian B, Van Teijlingen E, Mekkodathil A, Subramanya SH, Simkhada P. COVID-19 pandemic: public health implications in Nepal. *Nepal J Epidemiol*. (2020) 10:817–20. doi: 10.3126/nje.v10i1.28269
42. Bong CL, Brasher C, Chikumba E, Mcdougall R, Mellin-Olsen J, Enright A. The COVID-19 pandemic: effects on low and middle-income countries. *Anesth Analg*. (2020) 131:86–92. doi: 10.1213/ANE.0000000000004846
43. Shrestha A, Rajbhandari P, Bajracharya S. Hospital preparedness for outbreak at patan hospital: lesson learnt from COVID-19. *J Nepal Health Res Counc*. (2020) 18:142–3. doi: 10.33314/jnhrc.v18i1.2547
44. Shrestha GS, Paneru HR, Acharya SP, Shrestha SK, Sigdel MR, Tiwari S, et al. Preparedness for coronavirus disease in hospitals of Nepal: a nationwide survey. *JNMA J Nepal Med Assoc*. (2020) 58:248–51. doi: 10.31729/jnma.4941
45. Gompo TR, Shah BR, Karki S, Koirala P, Maharjan M, Bhatt DD. Risk factors associated with avian influenza subtype H9 outbreaks in poultry farms in Kathmandu valley, Nepal. *PLoS ONE*. (2020) 15:e0223550. doi: 10.1371/journal.pone.0223550
46. Mackenzie JS, Jeggo M. The one health approach-why is it so important? *Trop Med Infect Dis*. (2019) 4:88. doi: 10.3390/tropicalmed4020088
47. Alfano V, Ercolano S. The efficacy of lockdown against COVID-19: a cross-country panel analysis. *Appl Health Econ Health Policy*. (2020) 18:509–17. doi: 10.1007/s40258-020-00596-3
48. Kissler SM, Tedijanto C, Goldstein E, Grad YH, Lipsitch M. Projecting the transmission dynamics of SARS-CoV-2 through the postpandemic period. *Science*. (2020) 368:860–8. doi: 10.1126/science.abb5793

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

PERSPECTIVE
published: 11 August 2020
doi: 10.3389/fimmu.2020.02014

Understanding the Pathophysiology of COVID-19: Could the Contact System Be the Key?

Simone Meini[1], Andrea Zanichelli[2], Rodolfo Sbrojavacca[3], Federico Iuri[4], Anna Teresa Roberts[5], Chiara Suffritti[2] and Carlo Tascini[3]*

[1] Internal Medicine Unit, Azienda USL Toscana Centro, Santa Maria Annunziata Hospital, Florence, Italy, [2] General Medicine Unit, ASST Fatebenefratelli Sacco, Ospedale Luigi Sacco-Università degli Studi di Milano, Milan, Italy, [3] Infectious Diseases Clinic, Santa Maria Misericordia Hospital, Università degli Studi di Udine, Udine, Italy, [4] Department of Emergency, Santa Maria Misericordia Hospital, Università degli Studi di Udine, Udine, Italy, [5] Medical Department, Azienda USL Toscana Nord-Ovest, Pisa, Italy

To date the pathophysiology of COVID-19 remains unclear: this represents a factor determining the current lack of effective treatments. In this paper, we hypothesized a complex host response to SARS-CoV-2, with the Contact System (CS) playing a pivotal role in innate immune response. CS is linked with different proteolytic defense systems operating in human vasculature: the Kallikrein–Kinin (KKS), the Coagulation/Fibrinolysis and the Renin–Angiotensin (RAS) Systems. We investigated the role of the mediators involved. CS consists of Factor XII (FXII) and plasma prekallikrein (complexed to high-molecular-weight kininogen-HK). Autoactivation of FXII by contact with SARS-CoV-2 could lead to activation of intrinsic coagulation, with fibrin formation (microthrombosis), and fibrinolysis, resulting in increased D-dimer levels. Activation of kallikrein by activated FXII leads to production of bradykinin (BK) from HK. BK binds to B2-receptors, mediating vascular permeability, vasodilation and edema. B1-receptors, binding the metabolite [des-Arg^9]-BK (DABK), are up-regulated during infections and mediate lung inflammatory responses. BK could play a relevant role in COVID-19 as already described for other viral models. Angiotensin-Converting-Enzyme (ACE) 2 displays lung protective effects: it inactivates DABK and converts Angiotensin II (Ang II) into Angiotensin-(1-7) and Angiotensin I into Angiotensin-(1-9). SARS-CoV-2 binds to ACE2 for cell entry, downregulating it: an impaired DABK inactivation could lead to an enhanced activity of B1-receptors, and the accumulation of Ang II, through a negative feedback loop, may result in decreased ACE activity, with consequent increase of BK. Therapies targeting the CS, the KKS and action of BK could be effective for the treatment of COVID-19.

Keywords: SARS-CoV-2, COVID-19, pathophysiology, Contact System, bradykinin, ACE, coagulation

OPEN ACCESS

Edited by:
Gennady Bocharov, Institute of Numerical Mathematics (RAS), Russia

Reviewed by:
Klaus T. Preissner, University of Giessen, Germany
Zhenlong Liu, McGill University, Canada

****Correspondence:***
Simone Meini
simonemeini2@gmail.com

Specialty section:
This article was submitted to Viral Immunology, a section of the journal Frontiers in Immunology

Received: *16 April 2020*
Accepted: *24 July 2020*
Published: *11 August 2020*

Citation:
Meini S, Zanichelli A, Sbrojavacca R, Iuri F, Roberts AT, Suffritti C and Tascini C (2020) Understanding the Pathophysiology of COVID-19: Could the Contact System Be the Key? Front. Immunol. 11:2014. doi: 10.3389/fimmu.2020.02014

INTRODUCTION

Starting in December 2019 in Wuhan (Hubei Province, China), a novel coronavirus, designated SARS-CoV-2, has caused an international outbreak of a respiratory illness (COVID-19), rapidly evolving into a pandemic. The clinical spectrum of SARS-CoV-2 infection varies from asymptomatic or self-limiting mild forms, occurring in most cases, to severe progressive

pneumonia with acute respiratory distress syndrome (ARDS), and death. In a yet to be defined percentage of cases, after about one week, there is a sudden and unpredictable worsening of clinical conditions (1).

At present, there is no vaccine or pharmacological treatments of proven efficacy for COVID-19 (2) and further investigation on effective drugs is required to face the current pandemic. One factor determining the lack of effective treatments is that the pathophysiology of COVID-19 remains largely unclear.

In this review, we try to address the complex link between the pathophysiology of COVID-19 and the different proteolytic defense systems operating in human vasculature, investigating the role of the mediators involved and speculating on the possibility of pharmacological modulation.

CLINICAL AND LABORATORY FINDINGS IN PATIENTS WITH COVID-19

COVID-19 is mainly a respiratory illness, but a wide variety of clinical manifestations have been described, including the central nervous (3) and the digestive (4) systems. Most symptomatic COVID-19 patients display manifestations such as fever (98.6%), dry cough (59.4%), dyspnea (31.2%), myalgias (34.8%), sore throat (17.4%), diarrhea (10.1%), and other (5). Olfactory and gustatory dysfunctions are common symptoms, occurring in about 50% of patients and often presenting early in the clinical course (6, 7). Low blood pressure values are frequently observed in hospitalized patients: Wang et al. (5) in their cohort reported a median of mean arterial pressure values of 90 mmHg despite 31% of patients having a history of hypertension.

The predominant findings of lung Computed Tomography are images of bilateral, peripheral and basal ground-glass opacities, crazy-paving pattern, consolidations, often in association (8), and ultrasonography precociously demonstrates a lung interstitial syndrome (9). These findings are consistent with a lung injury characterized by increased permeability, leaky blood vessels and edema, and have been confirmed by the histopathological data obtained from the lungs of patients who died from COVID-19, showing diffuse alveolar damage with necrosis of alveolar lining cells, pneumocyte type 2 hyperplasia, linear intra-alveolar fibrin deposition and increased lung weight due to edema; in addition, thrombi in pulmonary arteries with a diameter of 1–2 mm, without complete luminal obstruction, and massive alveolar capillary microthrombi were observed (10).

Concerning laboratory findings, an increase of lactate dehydrogenase levels and lymphocytopenia are common. Elevated levels of serum ferritin, as commonly found in viral infections, are detected in most patients (11). Levels of interleukin-6 (IL-6) are typically in the upper limit of the reference range and appear to correlate with disease severity (12, 13). IL-6-induced high levels of C-reactive protein (CRP) are typically more related to bacterial rather than to viral infections (11): in COVID-19 patients CRP values are very variable. Even in non-critical patients, high D-dimer levels are found in most patients. Prothrombin time is often slightly increased.

Levels of inflammatory and coagulation biomarkers vary considerably among patients with COVID-19, suggesting the existence of different biochemical/clinical phenotypes, in which the predominant systems involved and the inflammatory and coagulopathy response patterns differ.

From a pathogenetic point of view, it is clear that a link (to date not yet fully clarified) exists between the clinical manifestations and alterations of the inflammatory and coagulation systems, and that these different systems are only apparently unrelated.

THE ROLE OF THE CONTACT SYSTEM IN THE PATHOPHYSIOLOGY OF COVID-19

The Contact System (CS) is part of the innate immune system and of inflammatory response mechanism against artificial material, misfolded and foreign proteins and microorganisms (including viruses), found in the intravascular compartment. It remains to be clarified whether contact factors bind and activate directly on the viral surface or on infected cells (14).

The main proteins of the CS are the Factor XII (FXII), the prekallikrein (PK) and the high-molecular-weight kininogen (HK). These proteins are produced by the liver and circulate as zymogens into the bloodstream. Virtually all plasma PK circulates in complex with HK.

Auto-activation of FXII to FXIIa by contact with a variety of artificial and biological negatively charged surfaces, including microorganisms, gives rise to CS cascade. Biological substances with the potential to support its activation include: DNA, RNA, polyphosphates retained on activated platelet surface, aggregated proteins, neutrophil extracellular traps (NETs) and ferritin (15–19). Kannemeier et al. (20) presented evidence that different forms of eukaryotic and prokaryotic RNA serve as promoters of blood coagulation, enhancing auto-activation of proteases of the CS, such as FXII and FXI. As the extracellular RNA derived from damaged or necrotic cells represented a "foreign surface" able to activate the CS, it could be speculated that the same process may be initiated by viral RNA. In addition, at times of cellular stress (i.e., hypoxia, hyperthermia, oxygen radical production) such as that observed during COVID-19, endogenous "alarmins" named "Danger-Associated Molecular Patterns" (DAMPs) are released from necrotic cells. These molecules are able to initiate appropriate defense reactions

Abbreviations: ACE, Angiotensin-Converting Enzyme; ACE2, Angiotensin-Converting Enzyme 2; Ang I, Angiotensin I; Ang II, Angiotensin II; Ang (1-7), Angiotensin 1-7; Ang (1-9), Angiotensin 1-9; ARB, angiotensin receptor blocker; ARDS, Acute respiratory distress syndrome; AT1R, Angiotensin II type 1 receptor; AT2R, Angiotensin II type 2 receptor; BK, Bradykinin; B1R, Bradykinin B1 receptor; B2R, Bradykinin B2 receptor; COVID-19, COronaVIrus Disease 2019; CS, Contact System; C1-INH, C1-inhibitor; DABK, [des-Arg^9]-Bradykinin; DAKD, [des-Arg^{10}]-Kallidin; DAMPs, Danger-Associated Molecular Patterns; DHF, dengue hemorrhagic fever; FXI, Factor XI; FXII, Factor XII; HK, High-molecular-weight Kininogen; HSV1, Herpes simplex virus type-1; IL-6, Interleukin-6; KAL, Kallikrein; KD, Kallidin; KKS, Kallikrein–Kinin System; LMWH, Low-Molecular Weight Heparin; PAMPs, Pathogen-Associated Molecular Patterns; PK, Prekallikrein; PRCP, Prolyl-carboxypeptidase; PRR, Pattern-Recognition Receptor; RAS, Renin–Angiotensin System; SARS-CoV-2, Severe Acute Respiratory Syndrome – Coronavirus – 2; TLR, Toll-like receptor; TNF-alpha, tumor necrosis factor-alpha; t-PA, tissue-Plasminogen Activator.

associated with "sterile" inflammation and tissue repair, engaging the "Pattern-Recognition Receptors" (PRRs), such as the cell membrane and endosomal Toll-like receptors (TLRs) (21). Moreover, during viral infections, TLRs represent a host primary line of defense for pathogen sensing, due to their properties to bind diverse exogenous ligands (the "Pathogen-Associated Molecular Patterns," PAMPs), including viral RNA (21); DAMPs and PAMPs are able to activate the FXII and the CS.

HK, complexed with PK, binds to these "surfaces": the domain 5 is the artificial surface-binding region of HK, while the domain 6 binds PK and FXI in order to initiate the intrinsic coagulation (16). After HK binds to a surface, PK is exposed to conversion to plasma kallikrein (KAL) by FXIIa: the binding induces a conformational change in PK so that it acquires enzymatic activity and can stoichiometrically cleave HK (22). In turn, KAL cleaves and activates more FXII, in a powerful positive feedback loop (14).

In addition, a vessel wall-associated serine protease, prolyl-carboxypeptidase (PRCP), is able to activate PK to KAL independent of FXIIa (16).

The CS is involved in inflammation and in coagulation: when sufficient amounts of FXII are activated, FXIIa also activates FXI (to FXIa), and the intrinsic (or contact) coagulation pathway can start, leading to subsequent thrombin activation and fibrin formation. KAL can influence the fibrinolytic pathway by activating plasminogen into plasmin, thus leading also to fibrin degradation (23). D-dimer is a soluble fibrin degradation product deriving from the plasmin-mediated degradation of cross-linked fibrin: it can therefore be considered a biomarker of concomitant activation of both coagulation and fibrinolysis (24).

It should be remembered that plasmin can also activate FXII (25), and that FXIIa can act as a plasminogen activator too (26): it has been speculated that in the very early stages of *in vivo* contact activation, when PK has yet to become activated, plasmin could have an initiating role (26), however it should be noted that plasmin is hardly present in plasma as an active protease due to the very effective action of its specific inhibitor, the α2-anti-plasmin: plasmin is protected from inactivation by this inhibitor only when bound to fibrin.

The coagulation cascade can be modernly considered as a component and one of the intravascular effectors of innate immunity (immunothrombosis) (27). It is debatable whether the main physiological function of FXII is the activation of the intrinsic coagulation pathway, or if to be a component of the CS should be considered its main physiological function. For physiological hemostasis to occur, FXII auto-activation is dispensable (21). The FXII-induced intrinsic coagulation pathway is involved in pathological thrombus formation but is not associated with abnormal hemostasis: FXII-deficient subjects present in fact a normal hemostatic capacity (28, 29). Challenging the concept of the coagulation balance, targeting FXII or its activator polyphosphate can provide protection from thromboembolic diseases (and modulate immunothrombosis) without interfering with hemostasis and increasing the risk of bleeding (14, 16, 18, 30, 31).

COVID-19 is a condition clearly characterized by coagulopathy, as testified by the extensive microthrombosis reported in lung autopsies (10), and the high levels of D-dimer displayed by most patients indirectly testify the hyperactivation of both coagulation and fibrinolysis, and overwhelming immunothrombosis. It should be remembered that low-molecular weight heparins (LMWHs) have been extensively used in hospitalized COVID-19 patients for preventing venous thromboembolism and thrombotic complications, and are currently investigated in randomized controlled trials (i.e., ClinicalTrials.gov Identifier: NCT04401293).

It is interesting to note that in the physiological state FXII acts as a growth factor promoting angiogenesis and wound repair (32), but pathologically it can promote lung fibroblast proliferation leading to pulmonary fibrosis (33): COVID-19 may also evolve into pulmonary fibrosis.

The archetypal contact activation disease state is sepsis from any etiology. There is no specific data on the model of SARS-CoV-2, but data may be gathered from other viral models. It is known that herpes simplex virus type-1 (HSV1) can trigger and amplify coagulation through the contact phase and intrinsic coagulation pathway: both an inhibitor of FXIIa (corn trypsin inhibitor), and anti-FXII, anti-KAL and anti-FXI antibodies were able to inhibit HSV1-initiated clotting (34). Moreover, PK and FXII levels are significantly lower in patients with dengue hemorrhagic fever (DHF), probably due to activation and consumption (35).

It has been mentioned that CS is part of the innate immune system: it is known that non-structural protein 3 (nsp3) of coronaviruses results able to block the host innate immune response (36), and other nsp play a role in evading host recognition (37).

THE KALLIKREIN–KININ SYSTEM

The Kallikrein–Kinin System (KKS) is mainly a host inflammatory response mechanism, and although KKS and CS overlap and interact in the intravascular compartment (plasma KAL is part of both systems), the use of the two terms has different implications. Activation of KKS finally leads to the liberation of bradykinin (BK), and plays an essential role in inflammation, but not in blood coagulation (16).

Upon activation by FXIIa, KAL cleaves HK, releasing from its domain 4 the nonapeptide bradykinin (BK-1-9 or BK) (38); BK is converted by a carboxypeptidase to [des-Arg^9]-BK (BK-1-8 or DABK), an active metabolite (39). During inflammation, plasmin potentiates the cleavage of HK by KAL, thus enhancing BK production (40).

BK and DABK bind to two pharmacologically distinct G protein-coupled receptors: the bradykinin B2 receptor (B2R), whose ligand is BK, and the B1 receptor (B1R), whose main agonist is DABK (39). The B2R is widely and constitutively expressed in mammalian cells (e.g., endothelial and smooth muscle cells), whereas the B1R is mostly inducible under the effect of cytokines during infections and immunopathology (41).

After binding through its B2R, BK activates signaling pathways resulting in increased vascular permeability, vasodilation, edema formation, hypotension, pain, fever

(14): all typical clinical features of COVID-19. BK is one of the most potent vasodilatory substances in humans: it is known that the BK-mediated angioedema is responsible for a very high percentage of serious morbidity and mortality (42). BK is also one of the most potent inflammatory mediators, able to stimulate the production of superoxide radicals and nitric oxide and to modulate the mobilization and release of histamine, arachidonic acid, prostaglandin E2, prostacyclin, pro-inflammatory interleukin-1, and tumor necrosis factor (TNF)-alpha (41). Thereafter, BK has shown to increase IL-6 production via B2R in colorectal cancer cell (43), and the B2R-antagonist icatibant was able to inhibit the BK-induced IL-6 release (44). This effect is interesting: also chloroquine, that has been extensively used and investigated for COVID-19 treatment, was able to reduce IL-6 production by monocytes/macrophages (45). BK also stimulates tissue plasminogen activator (t-PA) release from human endothelium through a B2R-dependent mechanism: this effect was significantly reduced in smokers (46). A strong link between KKS and the renin–angiotensin system (RAS) is testified by the fact that B2R forms homo- and heterodimers with several receptors of the RAS, that are important for some physiologic functions, including thrombosis risk regulation. The B2R also complexes with endothelial cell nitric oxide synthase, while the B1R couples with inducible nitric oxide synthase (16).

B1R mediates several responses including vasodilation, hypotension, and increased vascular permeability (41): all typical features of COVID-19.

Human kallikreins have been detected in many tissues (47), including the epithelia of the upper and lower respiratory tract: there are in fact two classical pathways for the generation of kinins, the plasma and the tissue KKS. As the substrate of plasma KAL is HK (leading to BK), the substrate of tissue kallikreins is the low-molecular-weight kininogen, leading to formation of the decapeptide Lys-bradykinin or kallidin (KD). A carboxypeptidase leads to the formation of the active metabolite [des-Arg^{10}]-KD (DAKD) from KD. KD mainly binds to B2R, while B1R has a high affinity for DAKD (48).

It is not known if SARS-CoV-2 infection is specifically associated with kinins dysregulation, but this happens in several viral models. Low levels of HK have been observed in DHF patients, perhaps due to proteolysis and generation of BK (49). Taylor et al. (50) previously described a novel mechanism of hantavirus-induced vascular leakage involving activation of the KKS, showing that incubation of FXII, PK, and HK with hantavirus-infected endothelial cells results in increased cleavage of HK, higher enzymatic activities of FXII/KAL and increased liberation of BK, that dramatically increased cell permeability. Furthermore, the alterations in permeability could be prevented using inhibitors directly blocking BK binding, the activity of FXII, or the activity of KAL (50). Infection of guinea pigs by nasal instillation of parainfluenza-3 virus induced airway hyperreactivity and influx of inflammatory cells into lung tissues, and these responses were attenuated by B2R-antagonists (51). Tissue kallikrein 1 was shown to intervene early during influenza infection, enhancing the antiviral defense, and the decreased expression observed in patients with chronic obstructive pulmonary disease could contribute to the less favorable evolution of influenza in this group (52).

Therefore, the KKS appears to be involved in vascular leakage and inflammatory response observed during different viral infections (14). We can speculate that modulation of the CS and the KKS may limit the evolution towards a frankly dysregulated host response also in SARS-CoV-2 infection.

Moreover, a role of BK in COVID-19 pathogenesis is suggested by several clinical features and symptoms observed in patients: given the close interconnection with the RAS, these aspects will be further discussed in the next chapter.

THE RENIN–ANGIOTENSIN SYSTEM (RAS) AND THE INTERPLAY WITH KKS

The renin–angiotensin system (RAS) is classically known for its effects on the cardiovascular system and fluid homeostasis, but it has become clear that the RAS is present in many tissues, where evidently has a role to play (53).

Starting from angiotensinogen, whose primary source is the liver, the RAS leads to the production of the multi-functional peptide hormone Angiotensin II (Ang II). Renin first catalyzes the cleavage of the peptide Angiotensin I (Ang I) from the N-terminus of the angiotensinogen molecule, then, sequentially, the dicarboxyl-peptidase angiotensin converting enzyme (ACE) removes two amino-acids from the C-terminus of Ang I to form Ang II (54). Ang II exerts its main functions binding to two specific G-protein coupled receptors: the ATII type 1 receptor (AT1R) and ATII type 2 receptor (AT2R) (54).

ACE is present in many tissues and is particularly abundant on the endothelium of the lungs: it is mainly anchored to the plasma membrane through a single trans-membrane domain, but a soluble form has also been described (53).

Apart from its well-known role as a peptidyl-dipeptidase forming Ang II, ACE is also described as a kininase II, able to inactivate BK, as well as KD (53). The affinity of ACE appears to be higher for BK than for Ang I, suggesting that ACE-inhibition may really involve the BK degradation more than the Ang II production (55). BK-evoked sensitization of airway sensory nerves is believed to be the main mechanism for ACE-inhibitor-induced dry cough (56): considering that dry cough is very frequently observed in COVID-19 patients, this pathway could in part explain the pathogenesis of this symptom. Additionally, a role of the BK has been hypothesized also for gustatory and olfactory dysfunctions (7); again, ACE-inhibitors can cause olfactory dysfunction (57).

In addition, over the last 20 years, knowledge of the biology and physiology of another enzyme besides ACE, the angiotensin converting enzyme 2 (ACE2), has accumulated (58): ACE2 is widely expressed, including type 2 alveolar epithelial cells, endothelial cells and enterocytes (10, 58).

Both ACE and ACE2 act as zinc metallopeptidases (ACE2 only acts as a carboxypeptidase), but differ for substrate specificities, displaying counterbalancing roles in the RAS.

ACE2 converts Ang I into Angiotensin (1-9), and Ang II into Angiotensin (1-7); unlike ACE, ACE2 does not cleave BK, and

is insensitive to conventional ACE-inhibitors (58). Ang II can be converted to angiotensin (1-7) also by PRCP in the low-pH areas of the kidney (59).

Angiotensin 1-7, acting on Mas receptor, exerts vasodilatory effects, thus diminishing and opposing the vasoconstrictor effect, mainly AT1R-mediated, of Ang II; moreover, it displays anti-fibrotic, anti-oxidant and anti-hypertrophic protective properties (58).

Therefore, ACE2 expression seems to protect from lung injury. Sodhi et al. (39) observed that a reduction in pulmonary ACE2 activity contributes to the pathogenesis of lung inflammation, resulting in prompt onset of neutrophil infiltration and more severe inflammation. Imai et al. (60) showed that the loss of ACE2 expression in acute lung injury leads to leaky pulmonary blood vessels through AT1R stimulation, while the AT2R protects against lung injury during sepsis. Angiotensin 1-9 has shown beneficial biological effects via the AT2R, resulting in protective effects on cardiac and vascular remodeling (58) and against pulmonary arterial hypertension, inflammation and fibrosis (61).

SARS-CoV-2 binds ACE2 for host cell entry, through the binding of its major spike glycoprotein (S1) to the N-terminal region of the receptor (62); chloroquine seems to interfere with ACE2 glycosylation, thus possibly preventing SARS-CoV-2 binding to target cells (63). Following binding with SARS-CoV-2, a loss of ACE2 function occurs, driven by endocytosis and activation of proteolytic cleavage and processing (58, 62). It can be assumed that this downregulation may be involved in the pathophysiology of COVID-19 and its manifestations. DABK is a substrate of ACE2, and the attenuation of ACE2 activity leads to impaired DABK inactivation and thus to enhanced B1R signaling.

In a mouse model, the lack of ACE2 function with consequent accumulation of Ang II, through a negative feedback loop,

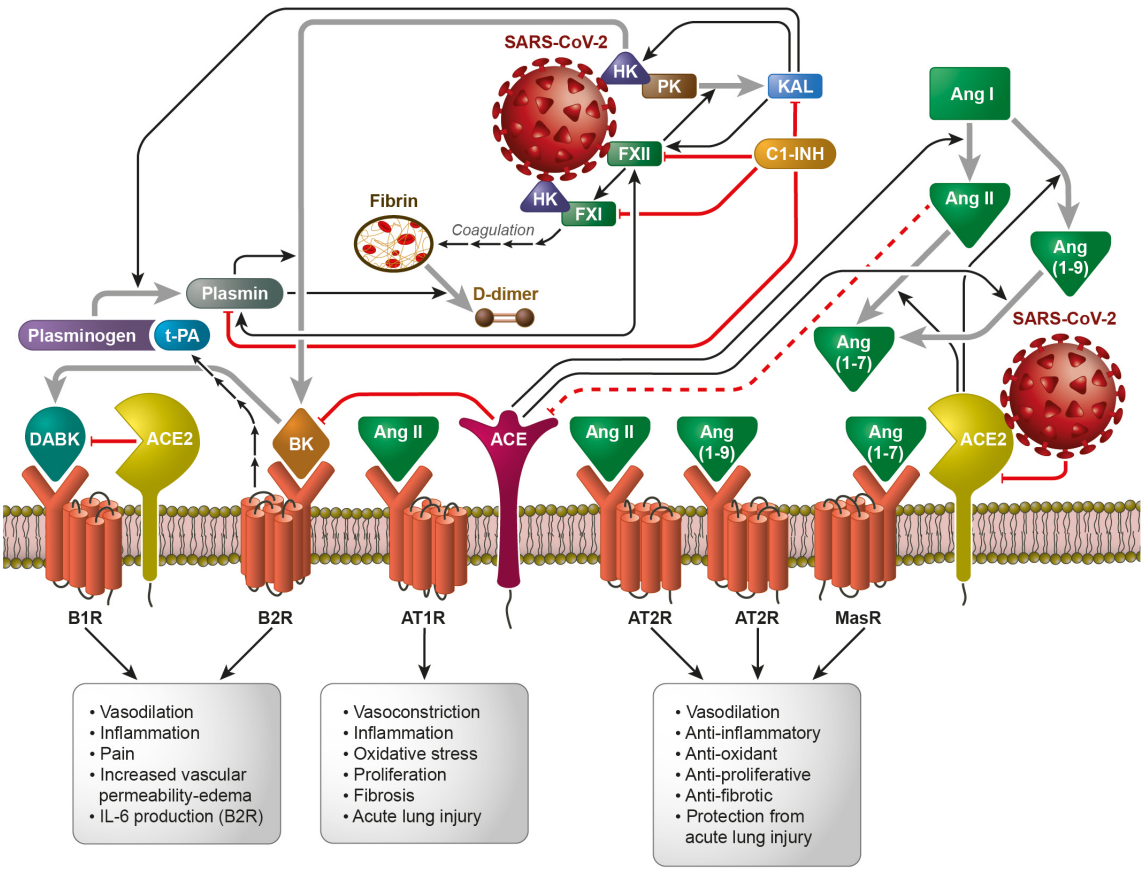


FIGURE 1 | The Contact System (CS) as a plausible link between Coagulation/Fibrinolysis, Kallikrein–Kinin and Renin–Angiotensin Systems in the pathobiology of SARS-CoV-2 infection (based on the hypothesis of the activation of FXII and HK by SARS-CoV-2, directly or following cell invasion and/or damage). Black arrows indicate enzymatic activation, while red lines represent inhibition or degradation/downregulation. The sequences of black arrows imply the involvement of other molecules not shown to activate the molecule indicated by the final arrow. Dashed red lines imply the involvement of other molecules not shown to inhibit the molecule indicated at the end of the line. Gray arrows indicate the transformation of one molecule into another. *ACE*, angiotensin converting enzyme; *ACE2*, angiotensin converting enzyme 2; *Ang I*, Angiotensin I; *Ang II*, Angiotensin II; *Ang* (1-7), Angiotensin 1-7; *Ang* (1-9), Angiotensin 1-9; *AT1R*, ATII type 1 receptor; *AT2R*, ATII type 2 receptor; *B1R*, B1 receptor; *B2R*, B2 receptor; *BK*, bradykinin; *C1-INH*, C1-inhibitor; *DABK*, [des-Arg^9]-BK; *FXI*, coagulation factor XI; *FXII*, coagulation factor XII; *HK*, high-molecular-weight kininogen; *IL-6*, interleukin-6; *KAL*, plasma kallikrein; *MasR*, Mas receptor; *PK*, plasma prekallikrein; *t-PA*, tissue plasminogen activator.

resulted in a secondary reduction of ACE activity (at the molecular level, Ang II downregulates renal ACE gene and enzymatic activity levels, as well as renin gene expression): these crosstalk effects between ACE2 and ACE appeared to be sex-dependent and more evident in males (64). It is known that COVID-19 affects male patients in a larger percentage (65) and with worse outcomes (66). The reduced activity of ACE is also expected to result in further BK accumulation. Moreover, it has been recognized *in vitro* that Ang II, through the stimulation of AT2R, is associated with increased expression of PRCP, leading to a KAL-mediated increased formation of BK (67).

Since SARS-CoV-2 binds to ACE2 receptors to enter host cells, and intravenous infusion of ACE-inhibitors and angiotensin receptor blockers (ARBs) in experimental animal models increased the amount of ACE2 receptors in the cardiopulmonary circulation, it has been speculated that patients chronically taking these drugs may be at increased risk of worse outcomes from COVID-19 (68). However, to date, there are no conclusive data demonstrating beneficial or adverse outcomes with background use of ACE-inhibitors, ARBs or other RAS antagonists among COVID-19 patients with a history of cardiovascular disease treated with these drugs (69–71). For the pathophysiological considerations previously made, however, in our opinion, it remains debatable if ACE-inhibitors, for their action on BK, should be temporarily suspended during the acute phase of illness, especially in the case of low blood pressure values.

Finally, it is interesting to observe that in an experimental malaria model (*Plasmodium* parasites during blood stages release kinins), exposure to captopril (an ACE-inhibitor that leads to the reduction of BK degradation) resulted in death in mice, while the concomitant administration of chloroquine protected them. B1R-knockout mice presented a significant reduction of survival when compared with wild-type mice, unlike the B2R-knockout ones (72). In this inflammation/infection model, chloroquine-induced upregulation of B1R expression proved protective: the full meaning of this result is unclear but might indicate that the selective inhibition of B2R could represent a rational modulation of dysregulated BK pathway during infection. Could the same considerations apply to COVID-19?

C1-INHIBITOR AND ITS POTENTIAL ROLE IN VIRAL INFECTIONS

Hereditary angioedema (HAE) represents the archetypal KKS disorder and can be due to a deficiency of C1-INH (Type 1), an abnormal C1-INH molecule (Type 2), or a gain-in-function of FXII with consequent plasma C1-INH consumption (Type 3) (73). Thrombin formation is not considered a feature of this disorder: even if patients with acute attacks have elevated D-dimer levels, they do not display an increased thrombotic risk (74). Clinical pictures of activation of CS and KKS without (such as HAE) and with thrombin formation (such as sepsis) can be in fact distinguished (16): COVID-19 evidently falls into the latter group.

C1-INH is a protein able to inhibit multiple serine proteases involved in the CS, KKS, Complement, Fibrinolysis, and Coagulation Systems: through the inhibition of C1r and C1s subcomponents of C1 complex, FXIIa, and KAL, C1-INH prevents the activation of CS and KKS. The N-terminal end (non-serpin domain) confers to C1-INH the capacity to bind lipopolysaccharides and E-selectin: owing to this moiety, C1-INH can also intervene in the regulation of inflammatory reactions

TABLE 1 | Potential therapeutic approaches able to modulate the systems involved in the pathogenesis of COVID-19.

Drug	Mechanism of action	Labeled indication	Potential role in COVID-19
Contact system			
C1-inhibitor	Inhibition of CS, Coagulation/Fibrinolytic systems, complement and KKS	Treatment and prevention of angioedema attacks in hereditary angioedema	Inhibition of all systems involved
Anti-factor XII (FXII) antibody	Monoclonal antibody inhibiting FXII	Phase II study ongoing; phase III study planned. Studied indication: prevention of angioedema attacks	Inhibition of FXII and consequently of CS and KKS
			Prevention and treatment of thrombosis, without increasing bleeding risk (Action on Coagulation system)
Kallikrein–kinin system			
Icatibant	Bradykinin type 2 receptor (B2R)-antagonist	Treatment of acute attacks in hereditary angioedema	Inhibition of pro-inflammatory and vasoactive actions of BK
Lanadelumab	Monoclonal antibody inhibiting plasma KAL	Prevention of attacks of hereditary angioedema	Inhibition of KKS and BK generation
Ecallantide	Inhibition of plasma KAL	Treatment of acute attacks in hereditary angioedema	Inhibition of KKS and BK generation
Coagulation system			
Low-Molecular-Weight Heparin/Fondaparinux	Catalyzed inhibition of activated coagulation factor X by antithrombin	Prevention and treatment of venous thromboembolism	Prevention and treatment of thrombosis and pulmonary embolism (consider bleeding risk)
Anti-factor XII (FXII) antibody	See above	See above	See above

(75). Moreover, C1-INH inhibits selectin-mediated leukocyte adhesion, regardless of its protease inhibitory activity (76).

Wygrecka et al. (77) showed that C1-INH is able to inhibit the cytotoxic activity of extracellular histones (that play a determining role in pulmonary injury leading to ARDS) and the release of several cytokines, such as TNF-alpha, IL-1b, and IL-6. It is interesting to note that accumulation of extracellular histones has been detected during infection due to influenza virus, and anti-histone antibodies have led to a marked decrease in the lung damage consisting of widespread pulmonary microvascular thrombosis, endothelial necrosis, hemorrhagic effusions and edema (78). These histopathological findings are observed also in COVID-19, although there are several differences compared to the influenza model (10) whose discussion goes beyond the scope of this review. Although there is actually no specific evidence regarding SARS-CoV-2 infection, it can be assumed that C1-INH might have beneficial effects also in this case, both through the inhibition of the CS and KKS, especially regarding the BK-induced vascular leakage and edema formation, and its anti-inflammatory activity mediated by inhibition of complement activation and histone toxicity.

Figure 1 shows the interconnection between the different human proteolytic systems operating in the vasculature, proposing a picture of an integrated host response to SARS-CoV-2 infection.

Table 1 lists some available drugs potentially representing effective therapeutic approaches in COVID-19, by modulation of the pathways and systems whose involvement has been hypothesized in its pathogenesis.

DISCUSSION AND CONCLUSION

The hypothesis of the involvement of different human proteolytic defense systems operating in the vasculature in the pathogenesis of COVID-19 has recently been proposed also by other authors. van de Veerdonk et al. (79) hypothesized that a kinin-dependent local lung angioedema via B1R and eventually B2R is an important feature of COVID-19 and proposed that blocking the B2R and inhibiting plasma KAL activity might be beneficial in early disease, preventing ARDS. Roche and Roche (80) emphasized the pivotal role of BK and DABK, suggesting that the B2R-antagonist icatibant might be able to interrupt the dysregulated pathway, thereby improving clinical outcomes. Colarusso et al. (23) proposed instead to block pharmacologically the KKS upstream of the BK, by means of lanadelumab. Regarding B1R-antagonists, several companies have in past developed orally available molecules, and some of these entered phase II clinical trials, but none have been developed further; possible reasons for this failure may be inefficacy in humans due to species differences, or human specific adverse effects (48).

In our opinion, the rational for modulating these pathways is strong but to date few data for COVID-19 are available. However, the exceptional nature of this pandemic and the lack of effective interventions of proven efficacy makes it necessary to explore further therapeutic possibilities.

Understanding the pathogenetic mechanisms underlying COVID-19 is crucial for the development of new effective therapeutic approaches modulating the CS, the KKS, the RAS and the Coagulation/Fibrinolysis System. The KKS inhibitors lanadelumab and ecallantide, licensed for the treatment of HAE, and several oral KKS inhibitors in clinical development, should be assessed for their efficacy in the treatment of patients with COVID-19. The same holds for icatibant, a selective B2R antagonist used for on demand treatment in HAE. Other promising CS-linked targets or mediators that should be explored in COVID-19 include anti-FXIIa antibodies and C1-INH. This pathophysiological therapeutic approach could be of great value also for other viral infections.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found at the appropriate doi link of every cited article.

AUTHOR CONTRIBUTIONS

SM, AZ, RS, FI, and CT: conceptualization. SM, AZ, RS, FI, CS, AR, and CT: formal analysis. AZ: funding acquisition. SM, AZ, and CT: investigation and project administration. SM, AZ, RS, FI, AR, CS, and CT: methodology and resources. SM, AZ, AR, and CT: writing – original draft preparation and writing – review and editing. All authors contributed to the article and approved the submitted version.

FUNDING

The CSL Behring funded the publishing support and journal styling services, but had no role in the conduct of the research, preparation of the article, in study design, in the collection, analysis and interpretation of data, in the writing of the report, and in the decision to submit the article for publication.

ACKNOWLEDGMENTS

We acknowledge SE*Ed* Medical Publishers, that provided publishing support and journal styling services.

REFERENCES

1. Cascella M, Rajnik M, Cuomo A, Dulebohn SC, Di Napoli R. *Features, Evaluation and Treatment Coronavirus (COVID-19)*. Treasure Island, FL: StatPearls Publishing (2020).
2. Lu H. Drug treatment options for the 2019-new Coronavirus (2019-nCoV). *Biosci Trends*. (2020) 14:69–71. doi: 10.5582/bst.2020.01020
3. Mao L, Jin H, Wang M, Hu Y, Chen S, He Q, et al. Neurologic manifestations of hospitalized patients with coronavirus disease 2019 in Wuhan, China. *JAMA Neurol*. (2020) 77:683–90. doi: 10.1001/jamaneurol.2020.1127

4. Jin X, Lian JS, Hu JH, Gao J, Zheng L, Zhang YM, et al. Epidemiological, clinical and virological characteristics of 74 cases of coronavirus-infected disease 2019 (COVID-19) with gastrointestinal symptoms. *Gut*. (2020) 69:1002–9. doi: 10.1136/gutjnl-2020-320926
5. Wang D, Hu B, Hu C, Zhu F, Liu X, Zhang J, et al. Clinical characteristics of 138 hospitalized patients with 2019 novel Coronavirus-infected pneumonia in Wuhan, China. *JAMA*. (2020) 323:1061–9. doi: 10.1001/jama.2020.1585
6. Tong JY, Wong A, Zhu D, Fastenberg JH, Tham T. The prevalence of olfactory and gustatory dysfunction in COVID-19 patients: a systematic review and meta-analysis. *Otolaryngol Head Neck Surg*. (2020) 163:3–11. doi: 10.1177/0194599820926473
7. Meini S, Suardi LR, Busoni M, Roberts AT, Fortini A. Olfactory and gustatory dysfunctions in 100 patients hospitalized for COVID-19: sex differences and recovery time in real-life. *Eur Arch Otorhinolaryngol*. (2020):1–5. doi: 10.1007/s00405-020-06102-8 [Epub ahead of print].
8. Zhu J, Zhong Z, Li H, Ji P, Pnag J, Li B, et al. CT imaging features of 4121 patients with COVID-19: a meta-analysis. *J Med Virol*. (2020) 92:891–902. doi: 10.1002/jmv.25910
9. Peng QY, Wang XT, Zhang LN. Chinese critical care ultrasound study group (CCUSG). Findings of lung ultrasonography of novel corona virus pneumonia during the 2019-2020 epidemic. *Intensive Care Med*. (2020) 46:849–50. doi: 10.1007/s00134-020-05996-6
10. Ackermann M, Verleden SE, Kuehnel M, Haverich A, Welte T, Laenger F, et al. Pulmonary vascular endothelialitis, thrombosis, and angiogenesis in Covid-19. *N Engl J Med*. (2020) 383:120–8. doi: 10.1056/NEJMoa2015432
11. Slaats J, Ten Oever J, van de Veerdonk FL, Netea MG. IL-1β/IL-6/CRP and IL-18/ferritin: distinct inflammatory programs in infections. *PLoS Pathog*. (2016) 12:e1005973. doi: 10.1371/journal.ppat.1005973
12. Wu C, Chen X, Cai Y, Xia J, Zhou X, Xu S, et al. Risk factors associated with acute respiratory distress syndrome and death in patients with Coronavirus disease 2019 Pneumonia in Wuhan, China. *JAMA Intern Med*. (2020) 180:1–11. doi: 10.1001/jamainternmed.2020.0994
13. Quartuccio L, Sonaglia A, Pecori D, Peghin M, Fabris M, Tascini C, et al. Higher levels of IL-6 early after tocilizumab distinguish survivors from non-survivors in COVID-19 pneumonia: a possible indication for deeper targeting IL-6. *J Med Virol*. (2020). doi: 10.1002/jmv.26149 [Epub ahead of print].
14. Oehmcke-Hecht S, Köhler J. Interaction of the human contact system with pathogens-an update. *Front Immunol*. (2018) 9:312. doi: 10.3389/fimmu.2018.00312
15. Golas A, Yeh CH, Pitakjakpipop H, Siedlecki CA, Vogler EA. A comparison of blood factor XII autoactivation in buffer, protein cocktail, serum, and plasma solutions. *Biomaterials*. (2013) 34:607–20. doi: 10.1016/j.biomaterials.2012.09.034
16. Schmaier AH. The contact activation and kallikrein/kinin systems: pathophysiologic and physiologic activities. *J Thromb Haemost*. (2016) 14:28–39. doi: 10.1111/jth.13194
17. Mailer RKW, Hänel L, Allende M, Renné T. Polyphosphate as a target for interference with inflammation and thrombosis. *Front Med (Lausanne)*. (2019) 6:76. doi: 10.3389/fmed.2019.00076
18. Maas C, Renné T. Coagulation factor XII in thrombosis and inflammation. *Blood*. (2018) 131:1903–9. doi: 10.1182/blood-2017-04-569111
19. Naudin C, Burillo E, Blankenberg S, Butler L, Renné T. Factor XII contact activation. *Semin Thromb Hemost*. (2017) 43:814–26. doi: 10.1055/s-0036-1598003
20. Kannemeier C, Shibamiya A, Nakazawa F, Trusheim H, Ruppert C, Markart P, et al. Extracellular RNA constitutes a natural procoagulant cofactor in blood coagulation. *Proc Natl Acad Sci USA*. (2007) 104:6388–93. doi: 10.1073/pnas.0608647104
21. Preissner KT, Herwald H. Extracellular nucleic acids in immunity and cardiovascular responses: between alert and disease. *Thromb Haemost*. (2017) 117:1272–82. doi: 10.1160/TH-16-11-0858
22. Kaplan AP, Joseph K. Pathogenic mechanisms of bradykinin mediated diseases: dysregulation of an innate inflammatory pathway. *Adv Immunol*. (2014) 121:41–89. doi: 10.1016/B978-0-12-800100-4.00002-7
23. Colarusso C, Terlizzi M, Pinto A, Sorrentino R. A lesson from a saboteur: high molecular weight kininogen (HMWK) impact in COVID-19. *Br J Pharmacol*. (2020). doi: 10.1111/bph.15154 [Epub ahead of print].
24. Favresse J, Lippi G, Roy PM, Chatelain B, Jacqmin H, Cate HT, et al. D-dimer: preanalytical, analytical, postanalytical variables, and clinical applications. *Crit Rev Clin Lab Sci*. (2018) 55:548–77. doi: 10.1080/10408363.2018.1529734
25. Kaplan AP, Austen KF. A prealbumin activator of prekallikrein. II. Derivation of activators of prekallikrein from active Hageman factor by digestion with plasmin. *J Exp Med*. (1971) 133:696–712. doi: 10.1084/jem.133.4.696
26. Maas C. Plasminflammation-an emerging pathway to bradykinin production. *Front Immunol*. (2019) 10:2046. doi: 10.3389/fimmu.2019.02046
27. Engelmann B, Massberg S. Thrombosis as an intravascular effector of innate immunity. *Nat Rev Immunol*. (2013) 13:34–45. doi: 10.1038/nri3345
28. Endler G, Marsik C, Jilma B, Schickbauer T, Quehenberger P, Mannhalter C. Evidence of a U-shaped association between factor XII activity and overall survival. *J Thromb Haemost*. (2007) 5:1143–8. doi: 10.1111/j.1538-7836.2007.02530.x
29. Gailani D, Renné T. Intrinsic pathway of coagulation and arterial thrombosis. *Arterioscler Thromb Vasc Biol*. (2007) 27:2507–13. doi: 10.1161/ATVBAHA.107.15595
30. Long AT, Kenne E, Jung R, Fuchs TA, Renné T. Contact system revisited: an interface between inflammation, coagulation, and innate immunity. *J Thromb Haemost*. (2016) 14:427–37. doi: 10.1111/jth.13235
31. Baeriswyl V, Calzavarini S, Chen S, Zorzi A, Bologna L, Angelillo-Scherrer A, et al. A synthetic Factor XIIa Inhibitor blocks selectively intrinsic coagulation initiation. *ACS Chem Biol*. (2015) 10:1861–70. doi: 10.1021/acschembio.5b00103
32. LaRusch GA, Mahdi F, Shariat-Madar Z, Adams G, Sitrin RG, Zhang WM, et al. Factor XII stim- ulates ERK1/2 and Akt through uPAR, integrins, and the EGFR to initiate angiogenesis. *Blood*. (2010) 115:5111–20. doi: 10.1182/blood-2009-08-236430
33. Wujak L, Didiasova M, Zakrzewicz D, Frey H, Schasfer L, Wygrecka M. Heparan sulfate proteoglycans mediate factor XIIa binding to the cell surface. *J Biol Chem*. (2015) 290:7027–39. doi: 10.1074/jbc.M114.606343
34. Gershom ES, Sutherland MR, Lollar P, Pryzdial EL. Involvement of the contact phase and intrinsic pathway in herpes simplex virus-initiated plasma coagulation. *J Thromb Haemost*. (2010) 8:1037–43. doi: 10.1111/j.1538-7836.2010.03789.x
35. Edelman R, Nimmannitya S, Colman RW, Talamo RC, Top FH Jr. Evaluation of the plasma kinin system in dengue hemorrhagic fever. *J Lab Clin Med*. (1975) 86:410–21.
36. Lei J, Kusov Y, Hilgenfeld R. Nsp3 of coronaviruses: structures and functions of a large multi-domain protein. *Antiviral Res*. (2018) 149:58–74. doi: 10.1016/j.antiviral.2017.11.001
37. Deng X, Baker SC. An "Old" protein with a new story: Coronavirus endoribonuclease is important for evading host antiviral defenses. *Virology*. (2018) 517:157–63. doi: 10.1016/j.virol.2017.12.024
38. Colman RW, Schmaier AH. Contact system: a vascular biology modulator with anticoagulant, profibrinolytic, antiadhesive, and proinflammatory attributes. *Blood*. (1997) 90:3819–43.
39. Sodhi CP, Wohlford-Lenane C, Yamaguchi Y, Prindle T, Fulton WB, Wang S, et al. Attenuation of pulmonary ACE2 activity impairs inactivation of des-Arg9 bradykinin/BKB1R axis and facilitates LPS-induced neutrophil infiltration. *Am J Physiol Lung Cell Mol Physiol*. (2018) 314:L17–31. doi: 10.1152/ajplung.00498.2016
40. Kleniewski J, Blankenship DT, Cardin AD, Donaldson V. Mechanism of enhanced kinin release from high molecular weight kininogen by plasma kallikrein after its exposure to plasmin. *J Lab Clin Med*. (1992) 120:129–39.
41. Marceau F, Bachelard H, Bouthillier J, Fortin JP, Morissette G, Bawolak MT, et al. Bradykinin receptors: agonists, antagonists, expression, signaling, and adaptation to sustained stimulation. *Int Immunopharmacol*. (2020) 82:106305. doi: 10.1016/j.intimp.2020.106305
42. Cicardi M, Zuraw BL. Angioedema due to bradykinin dysregulation. *J Allergy Clin Immunol Pract*. (2018) 6:1132–41. doi: 10.1016/j.jaip.2018.04.022
43. Wang G, Ye Y, Zhang X, Song J. Bradykinin stimulates IL-6 production and cell invasion in colorectal cancer cells. *Oncol Rep*. (2014) 32:1709–14. doi: 10.3892/or.2014.3366
44. Paegelow I, Werner H, Vietinghoff G, Wartner U. Release of cytokines from isolated lung strips by bradykinin. *Inflamm Res*. (1995) 44:306–11. doi: 10.1007/bf02032574

45. Jang CH, Choi JH, Byun MS, Jue DM. Chloroquine inhibits production of TNF-alfa, IL-1beta and IL-6 from lipopolysaccharide-stimulated human monocytes/macrophages by different modes. *Rheumatology*. (2006) 45:703–10. doi: 10.1093/rheumatology/kei282
46. Pretorius M, Rosenbaum DA, Lefebvre J, Vaughan DE, Brown NJ. Smoking impairs bradykinin-stimulated t-PA release. *Hypertension*. (2002) 39:767–71. doi: 10.1161/hy0302.105767
47. Petraki CD, Papanastasiou PA, Karavana VN, Diamandis EP. Cellular distribution of human tissue kallikreins: immunohistochemical localization. *Biol Chem*. (2006) 387:653–63.
48. Qadri F, Bader M. Kinin B1 receptors as a therapeutic target for inflammation. *Expert Opin Ther Targets*. (2018) 22:31–44. doi: 10.1080/14728222.2018.1409724
49. Ray S, Srivastava R, Tripathi K, Vaibhav V, Patankar S, Srivastava S. Serum proteome changes in dengue virus-infected patients from a dengue-endemic area of India: towards new molecular targets? *OMICS*. (2012) 16:527–36. doi: 10.1089/omi.2012.0037
50. Taylor SL, Wahl-Jensen V, Copeland AM, Jahrling PB, Schmaljohn CS. Endothelial cell permeability during hantavirus infection involves factor XII-dependent increased activation of the kallikrein-kinin system. *PLoS Pathog*. (2013) 9:e1003470. doi: 10.1371/journal.ppat.1003470
51. Broadley KJ, Blair AE, Kidd EJ, Bugert JJ, Ford WR. Bradykinin-induced lung inflammation and bronchoconstriction: role in parainfluenze-3 virus-induced inflammation and airway hyperreactivity. *J Pharmacol Exp Ther*. (2010) 335:681–92. doi: 10.1124/jpet.110.171876
52. Magnen M, Gueugnon F, Petit-Courty A, Baranek T, Sizaret D, Brewah YA, et al. Tissue kallikrein regulates alveolar macrophage apoptosis early in influenza virus infection. *Am J Physiol Lung Cell Mol Physiol*. (2019) 316:L1127–40. doi: 10.1152/ajplung.00379.2018
53. Lazartigues E, Feng Y, Lavoie JL. The two fACEs of the tissue renin-angiotensin systems: implication in cardiovascular diseases. *Curr Pharm Des*. (2007) 13:1231–45. doi: 10.2174/138161207780618911
54. Sparks MA, Crowley SD, Gurley SB, Mirotsou M, Coffman TM. Classical renin-angiotensin system in kidney physiology. *Compr Physiol*. (2014) 4:1201–28. doi: 10.1002/cphy.c130040
55. Taddei S, Bortolotto L. Unraveling the pivotal role of bradykinin in ACE inhibitor activity. *Am J Cardiovasc Drugs*. (2016) 16:309–21. doi: 10.1007/s40256-016-0173-4
56. Dicpinigaitis PV. Angiotensin-converting enzyme inhibitor-induced cough: ACCP evidence-based clinical practice guidelines. *Chest*. (2006) 129:169S–73S. doi: 10.1378/chest.129.1_suppl.169S
57. Scangas GA, Bleier BS. Anosmia: differential diagnosis, evaluation, and management. *Am J Rhinol Allergy*. (2017) 31:e3–7. doi: 10.2500/ajra.2017.31.4403
58. Gheblawi M, Wang K, Viveiros A, Nguyen Q, Zhong JC, Turner AJ, et al. Angiotensin-Converting enzyme 2: SARS-CoV-2 receptor and regulator of the renin-angiotensin system: celebrating the 20th anniversary of the discovery of ACE2. *Circ Res*. (2020) 126:1456–74. doi: 10.1161/CIRCRESAHA.120.317332
59. Grobe N, Weir NW, Leiva O, Ong FS, Bernstein KE, Schmaier AH, et al. Identification of Prolyl carboxypeptidase as an alternative enzyme for processing of renal angiotensin II using mass spectrometry. *Am J Physiol Cell Physiol*. (2013) 304:C945–53. doi: 10.1152/ajpcell.00346.2012
60. Imai Y, Kuba K, Rao S, Huan Y, Guo F, Guan B, et al. Angiotensin-converting enzyme 2 protects from severe acute lung failure. *Nature*. (2005) 436:112–6. doi: 10.1038/nature03712
61. Cha SA, Park BM, Kim SH. Angiotensin-(1-9) ameliorates pulmonary arterial hypertension via angiotensin type II receptor. *Korean J Physiol Pharmacol*. (2018) 22:447–56. doi: 10.4196/kjpp.2018.22.4.447
62. Hoffmann M, Kleine-Weber H, Schroeder S, Krüger N, Herrler T, Erichsen S, et al. SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor. *Cell*. (2020) 181:271–80.e8. doi: 10.1016/j.cell.2020.02.052
63. Devaux CA, Rolain JM, Colson P, Raoult D. New insights on the antiviral effects of chloroquine against coronavirus: what to expect for COVID-19? *Int J Antimicrob Agents*. (2020) 55:105938. doi: 10.1016/j.ijantimicag.2020.105938
64. Clotet-Freixas S, Soler MJ, Palau V, Anguiano L, Gimeno J, Konvalinka A, et al. Sex dimorphism in ANGII-mediated crosstalk between ACE2 and ACE in diabetic nephropathy. *Lab Invest*. (2018) 98:1237–49. doi: 10.1038/s41374-018-0084-x
65. Li LQ, Huang T, Wang YQ, Wang ZP, Liang Y, Huang TB, et al. 2019 novel coronavirus patients' clinical characteristics, discharge rate, and fatality rate of meta-analysis. *J Med Virol*. (2020) 92:577–83. doi: 10.1002/jmv.25757
66. Onder G, Rezza G, Brusaferro S. Case-fatality rate and characteristics of patients dying in relation to COVID-19 in Italy. *JAMA*. (2020) 323:1775–6. doi: 10.1001/jama.2020.4683
67. Zhu L, Carretero OA, Liao TD, Harding P, Li H, Sumners C, et al. Role of prolylcarboxypeptidase in angiotensin II type 2 receptor-mediated bradykinin release in mouse coronary artery endothelial cells. *Hypertension*. (2010) 56:384–90. doi: 10.1161/HYPERTENSIONAHA.110.155051
68. Diaz JH. Hypothesis: angiotensin-converting enzyme inhibitors and angiotensin receptor blockers may increase the risk of severe COVID-19. *J Travel Med*. (2020) 27:taaa041. doi: 10.1093/jtm/taaa041
69. Danser AHJ, Epstein M, Batlle D. Renin-angiotensin system blockers and the COVID-19 pandemic: at present there is no evidence to abandon renin-angiotensin system blockers. *Hypertension*. (2020) 75:1382–5. doi: 10.1161/HYPERTENSIONAHA.120.15082
70. Reynolds HR, Adhikari S, Pulgarin C, Troxel AB, Iturrate E, Johnson SB, et al. Renin-angiotensin-aldosterone system inhibitors and risk of Covid-19. *N Engl J Med*. (2020) 382:2441–8. doi: 10.1056/NEJMoa2008975
71. Mancia G, Rea F, Ludergnani M, Apolone G, Corrao G. Renin-angiotensin-aldosterone system blockers and the risk of Covid-19. *N Engl J Med*. (2020) 382:2431–40. doi: 10.1056/NEJMoa2006923
72. Ventura PDS, Carvalho CPF, Barros NMT, Martins-Silva L, Dantas EO, Martinez C, et al. Malaria infection promotes a selective expression of kinin receptors in murine liver. *Malar J*. (2019) 18:213. doi: 10.1186/s12936-019-2846-3
73. Busse PJ, Christiansen SC. Hereditary angioedema. *N Engl J Med*. (2020) 382:1136–48. doi: 10.1056/NEJMra1808012
74. Reshef A, Zanichelli A, Longhurst H, Relan A, Hack CE. Elevated D-dimers in attacks of hereditary angioedema are not associated with increased thrombotic risk. *Allergy*. (2015) 70:506–13. doi: 10.1111/all.12587
75. Cicardi M, Zingale L, Zanichelli A, Pappalardo E, Cicardi B. C1 inhibitor: molecular and clinical aspects. *Springer Semin Immunopathol*. (2005) 27:286–98. doi: 10.1007/s00281-005-0001-4
76. Cai S, Dole VS, Bergmeier W, Scafidi J, Feng H, Wagner DD, et al. A direct role for C1 inhibitor in regulation of leukocyte adhesion. *J Immunol*. (2005) 174:6462–6. doi: 10.4049/jimmunol.174.10.6462
77. Wygrecka M, Kosanovic D, Wujak L, Reppe K, Henneke I, Frey H, et al. Antihistone properties of C1 esterase inhibitor protect against lung injury. *Am J Respir Crit Care Med*. (2017) 196:186–99. doi: 10.1164/rccm.201604-0712OC
78. Ashar HK, Mueller NC, Rudd JM, Snider TA, Achanta M, Prasanthi M, et al. The role of extracellular histones in influenza virus pathogenesis. *Am J Pathol*. (2018) 188:135–48. doi: 10.1016/j.ajpath.2017.09.014
79. van de Veerdonk FL, Netea MG, van Deuren M, van der Meer JW, de Mast O, Brüggemann RJ, et al. Kallikrein-kinin blockade in patients With COVID-19 to prevent acute respiratory distress syndrome. *Elife*. (2020) 9:e57555. doi: 10.7554/eLife.57555
80. Roche JA, Roche R. A hypothesized role for dysregulated bradykinin signaling in COVID-19 respiratory complications. *FASEB J*. (2020) 00:1–5. doi: 10.1096/fj.202000967

Conflict of Interest: AZ received speaker/consultancy fees and/or was a member of medical/advisory boards for CSL Behring, Shire/Takeda, and SOBI. All the authors declare that they have no financial competing interests about the topic of this article, except for the publishing support and journal styling services, that were provided by SEEd Medical Publishers and funded by CSL Behring, Italy.

frontiers in Medicine

ORIGINAL RESEARCH
published: 11 August 2020
doi: 10.3389/fmed.2020.00485

Clinical Characteristics and Prognosis of 218 Patients With COVID-19: A Retrospective Study Based on Clinical Classification

*Xiquan Yan[1,2,3], Xiaotong Han[1], Danhong Peng[4], Yong Fan[5], Zhixiong Fang[6], Da Long[7], Yu Xie[8], Shuibo Zhu[9], Fang Chen[1], Wei Lin[1] and Yimin Zhu[1,2]**

[1] Hunan Provincial Institute of Emergency Medicine, Hunan Provincial People's Hospital/The First Affiliated Hospital, Hunan Normal University, Changsha, China, [2] School of Life Sciences, Hunan Normal University, Changsha, China, [3] Xiangtan Central Hospital, Xiangtan, China, [4] Department of Infectious Diseases, Shaoyang Central Hospital, Shaoyang, China, [5] Department of Infectious Diseases, Loudi Central Hospital, Loudi, China, [6] Public Health Centre, Xiangtan Central Hospital, Xiangtan, China, [7] Department of Science and Education, Shaoyang Central Hospital, Shaoyang, China, [8] Department of Critical Care Medicine, Loudi Central Hospital, Loudi, China, [9] Department of Hepatobiliary Surgery, Loudi Central Hospital, Loudi, China

OPEN ACCESS

Edited by:
Zisis Kozlakidis, International Agency for Research on Cancer (IARC), France

Reviewed by:
Huriye Berk Takir, Health Sciences University Süreyyapaşa Chest Diseases and Thoracic Surgery Training and Research Hospital, Turkey
Yimin Li, Guangzhou Medical University, China

***Correspondence:**
Yimin Zhu
zhuyimincs@163.com

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: 20 April 2020
Accepted: 17 July 2020
Published: 11 August 2020

Citation:
Yan X, Han X, Peng D, Fan Y, Fang Z, Long D, Xie Y, Zhu S, Chen F, Lin W and Zhu Y (2020) Clinical Characteristics and Prognosis of 218 Patients With COVID-19: A Retrospective Study Based on Clinical Classification. Front. Med. 7:485. doi: 10.3389/fmed.2020.00485

Background: Coronavirus disease 2019 (COVID-19) is an emerging infectious disease that has spread worldwide.

Methods: This was a retrospective case series involving 218 patients admitted to three tertiary hospitals in the Loudi, Shaoyang, and Xiangtan areas of China from January 21 to June 27, 2020, who were confirmed by RT-PCR to have SARS-CoV-2. The patients' clinical characteristics, laboratory results, treatments, and prognoses based on clinical classification were recorded. Poor outcome was defined as admission to an ICU, the use of mechanical ventilation, or death.

Results: The patients were classified into four clinical groups based on disease severity, namely mild (10/218, 5%), moderate (146/218, 67%), severe (24/218, 11%), or critical (14/218, 6%); 24 (11%) asymptomatic cases were also included in the study. The most common symptoms were self-reported cough (162/218, 74%), fever (145/218, 67%), sputum production (99/218, 45%), and fatigue (77/218, 35%). Among the 218 patients, 192 (88%) received lopinavir/ritonavir and interferon-alpha inhalation, and 196 (90%) patients received traditional Chinese medicine. Among the severe and critical patients, 25 (11%) were admitted to an ICU with or without mechanical ventilation, and one patient died. The presence of diabetes [relative risk (RR), 3.0; 95% CI, 1.3–6.8; $p = 0.007$) or other comorbidities (RR, 5.9; 95% CI, 1.9–17.8; $p = 0.002$) was independently associated with poor outcome. To date, 20 (9%) patients have retested positive for SARS-CoV-2 RNA after recovering and being discharged.

Conclusion: The majority of patients in this case series were clinically classified as having moderate COVID-19. Older patients tended to present with greater levels of clinical severity. The prognosis for patients who were elderly or had diabetes or other chronic comorbidities was relatively poor.

Keywords: COVID-19, clinical classification, clinical characteristics, treatment, prognosis

INTRODUCTION

Coronavirus disease 2019 (COVID-19) is caused by the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) and has rapidly spread across the world since first emerging in December 2019 (1). By April 17, 2020, COVID-19 had been discovered in 212 countries or territories, affecting 2,074,529 individuals and causing 139,378 deaths (2). The pandemic continues to escalate rapidly (3, 4). Typical symptoms are fever, cough, fatigue, and sputum production (5–7). However, a few patients with SARS-CoV-2 develop severe pneumonia, pulmonary edema, acute respiratory distress syndrome (ARDS), multiple organ failure, or even death (8–10).

In this retrospective case series, 218 patients testing positive for SARS-CoV-2 were clinically classified (mild, moderate, severe, or critical) according to the guidelines of the *Diagnosis and Treatment Protocol for COVID-19 (trial version 7)* issued by the National Health Commission of the People's Republic of China (11). Asymptomatic patients, who acquire and can transmit the coronavirus that causes COVID-19 (12, 13), were also included in this study.

These clinical classifications of COVID-19 are characterized by different clinical features and provide an objective basis for treatment and prognosis. To date, there have been no studies reporting COVID-19 treatment and outcomes based on clinical classification. Here, we comprehensively explored the clinical features, treatment, and prognosis of 218 confirmed SARS-CoV-2-infected patients in three top-tier hospitals in the Hunan province of China.

MATERIALS AND METHODS

Study Design and Participants

This multicenter, retrospective, and observational study was conducted on COVID-19 patients who were diagnosed in the Hunan province of China. Clinicians collected the patients who met the study inclusion criteria across three tertiary hospitals in the cities of Shaoyang, Loudi, and Xiangtan. The authors of this paper include the physicians who either supervised patient care or directly provided patient care for all of the patients included in the study to ensure complete follow-through for all cases.

We retrospectively analyzed COVID-19 patients who had been diagnosed during the period of January 21 to June 27, 2020, according to the WHO interim guidance. Real-time, reverse-transcription polymerase chain reaction (RT-PCR) tests for SARS-CoV-2 nucleic acids were performed on nasopharyngeal swabs from suspected patients to confirm the diagnosis. A confirmed case of COVID-19 was defined as having a positive result from the RT-PCR assay of a nasopharyngeal swab. Only laboratory-confirmed cases were included in the analysis. Suspected patients showing negative results after multiple tests during hospitalization were excluded. Where the typical symptoms, signs, and imaging manifestations were present, combined with a PaO_2/FiO_2 ratio <300 mmHg [based on the Berlin definition (14)], the patients were diagnosed as having ARDS. This study was approved by the ethics committee of each participating hospital. Written informed consent was obtained from all patients.

Clinical Classification

In this retrospective study, the whole disease course was examined for each patient. The clinical classification of the patients was based on the clinical conditions present during the most severe stage of COVID-19 based on the guidelines outlined in the *Diagnosis and treatment protocol for COVID-19* (trial version 7) released by the National Health Commission of the People's Republic of China on March 3, 2020 (http://www.nhc.gov.cn/xcs/zhengcwj/202003/46c9294a7dfe4cef80dc7f5912eb1989.shtml) (11). According to their clinical symptoms, signs, and chest imaging manifestations, the patients were classified as being mild, moderate, severe, or critical COVID-19 cases (see **Supplementary Material** for further details).

Data Collection

Data on the clinical characteristics, treatment, and prognosis of the 218 confirmed COVID-19 patients were collected at Shaoyang Central Hospital, Loudi Central Hospital, and Xiangtan Central Hospital in the Hunan province. The information of interest included age, sex, exposure history, smoking history, chronic diseases (including diabetes), symptoms from onset to hospital admission, laboratory tests on admission, coexisting infections, treatment, and living status. The data regarding the PaO_2/FiO_2 ratios were analyzed when the patients were monitored in the ICU.

Treatment

The patient treatment venue was determined based on the severity of each patient's disease according to the *Diagnosis and treatment protocol for COVID-19* (11). Suspected and confirmed cases were isolated and treated at designated hospitals with effective isolation, protection, and prevention conditions. Suspected cases were treated in isolation or together in a single room. Confirmed cases were treated in isolation or together in a single room. In the absence of pathogen-specific interventions, patient management largely depended on supportive treatment.

Most patients were provided with effective oxygen therapy, including a nasal catheter, mask oxygenation, and nasal high-flow oxygen therapy. Lopinavir/ritonavir, interferon-alpha inhalation, and arbidol were used as antiviral therapies. Moxifloxacin and other antibiotics were used to fight against bacterial infections where present. Glucocorticoids were used for short periods when patients showed rapidly progressive deterioration.

Patients who met the following criteria were admitted to the ICU for comprehensive treatment and care at an early stage: (1) severe cases with respiratory distress ($\geq$30 breaths/min) and chest imaging showing $>$50% of lung area with obvious lesion progression within 24–48 h; and (2) all critical cases.

In addition, patients were treated with traditional Chinese medicine (Qingfei Paidu decoction, Lianhuaqingwen capsules, Huoxiangzhengqi liquid, and/or Xuebijing injection) according

to the national guidelines. The full treatment protocol used for the COVID-19 patients is described in detail in the **Supplementary Materials**.

Discharge

When a patient's body temperature had returned to normal for more than 3 days, respiratory symptoms were significantly improved, pulmonary imaging showed obvious absorption of inflammation, and two consecutive SARS-CoV-2 nucleic acid tests were negative using respiratory tract samples (sampling interval of at least 24 h), he or she was discharged from the hospital. After discharge, the patients were required to quarantine and monitor their health for 14 days and requested to come back to the hospital for follow-up exams every 2–4 weeks.

Prognosis

All patients were traced from hospital admission to presenting prognosis. The primary outcome was "cured and discharged," and a poor outcome was defined as admission to an ICU, the use of mechanical ventilation, or death. This analysis method was referenced from other retrospective studies on viral pneumonia, such as SARS (15, 16). Time to discharge, time to death, and time to a poor outcome were analyzed using survival analysis (details in *Statistical Analysis*) tracing all patients from hospital admission to presenting prognosis.

Statistical Analysis

Data are presented as the mean ± standard deviation (SD) or median ± interquartile range (IQR) for continuous variables and as a number (%) for categorical variables. Differences in measurement data among the asymptomatic, mild, moderate,

TABLE 1 | Demographics and baseline characteristics of patients with COVID-19.

	All patient (n = 218)	Asymptomatic cases (n = 24)	Mild cases (n = 10)	Moderate cases (n = 146)	Severe cases (n = 24)	Critical cases (n = 14)	P-value
Age, years	42.9 (32.0–52.3)	32.0 (16.3–44.8)[c,d,e]	23.6 (11.8–34.3)[c,d,e]	42.3 (32.0–50.0)[a,b,d,e]	55.9 (46.3–67.0)[a,b,c]	59.5 (42.3–76.5)[a,b,c]	0.000*
Age range, years							
0–17	14 (6%)	6 (25%)	4 (40%)	4 (3%)	0	0	0.000**
18–39	82 (38%)	11 (46%)	5 (50%)	60 (41%)	4 (17%)	2 (14%)	
40–59	86 (39%)	6 (25%)	1 (10%)	65 (45%)	9 (37%)	5 (36%)	
60–79	30 (14%)	1 (4%)	0	15 (10%)	10 (42%)	4 (29%)	
≥80	6 (3%)	0	0	2 (1%)	1 (4%)	3 (21%)	
Sex							
Male	122 (56%)	16 (67%)	6 (60%)	77 (53%)	14 (58%)	9 (64%)	0.691
Female	96 (44%)	8 (33%)	4 (40%)	69 (47%)	10 (42%)	5 (36%)	
Exposure							
Exposure to Wuhan	111 (51%)	12 (50%)	3 (30%)	76 (52%)	13 (54%)	7 (50%)	0.768
Exposure to patients[†]	100 (46%)	18 (75%)	7 (70%)	59 (40%)	8 (33%)	8 (57%)	0.006**
Use of public transportation[‡]	4 (2 %)	0	0 (%)	3 (2%)	0	1 (7%)	0.535
Current smoking	23 (11%)	2 (8%)	2 (20%)	14 (10%)	3 (13%)	2 (14%)	0.902
Chronic medical illness							
Cardiovascular disease	38 (17%)	3 (13%)	0	17 (12%)	13 (54%)	5 (36%)	0.000**
Diabetes	27 (12%)	3 (13%)	0	12 (8%)	10 (42%)	2 (14%)	0.001**
Chronic pulmonary disease	14 (6%)	0	0	5 (3%)	4 (17%)	5 (36%)	0.000**
Liver disease	13 (6%)	1 (4%)	0	10 (7%)	2 (8%)	0	0.909
Malnutrition[§]	10 (5%)	0	1 (10%)	6 (4%)	1 (4%)	2 (14%)	0.193
Cerebrovascular disease	6 (3%)	0	0	2 (1%)	1 (4%)	3 (21%)	0.014**
Chronic renal diseases	4 (2%)	0	0	1 (1%)	1 (4%)	2 (14%)	0.031**
Cancer	2 (1%)	0	0	2 (1%)	0	0	1.000
Autoimmune disease	2 (1%)	0	0	1 (1%)	0	1 (7%)	0.256

Data are median (IQR), n (%), or mean (SD), unless otherwise specified.

**ANOVA was used for group comparisons with LSD for post-hoc tests.*

[a]vs. Asymptomatic cases ($p < 0.05$), [b]vs. Mild cases ($p < 0.05$), [c]vs. Moderate cases ($p < 0.05$), [d]vs. Severe cases ($p < 0.05$), [e]vs. Critical cases ($p < 0.05$).

***Statistical analysis was performed with the chi-square test or Fisher's exact test.*

[†] Patients who have confirmed SARS-CoV-2 infection or are highly suspected of being infected.

[‡] Without exposure to Wuhan and diagnosed patients.

[§] In this cohort, 3 patients suffer from undernutrition and 7 are overweight.

severe, and critical cases were compared with analysis of variance using the least significant difference *post-hoc* test. The Chi-square test and Fisher's exact test were used for categorical variables. Kaplan–Meier plots were used to analyze the survival data. Differences among groups of time-to-event data were determined using the Cox proportional hazards model, with graphical and statistical checks for the proportionality of hazards. Given that there were only 25 patients with poor outcomes in our study, we considered only three binary variables in the multiple regression model as *a priori* hypotheses: age of 60 years or older, diabetes, and other comorbidities. We used SPSS (version 26.0) for all analyses. For all analyses, $p < 0.05$ was considered statistically significant.

RESULTS

Demographics

A total of 218 patients were confirmed during the study period. The patients' demographic details and comorbidities are listed in **Table 1**. Age was correlated with the clinical classification of COVID-19 severity (**Figure 1**). The median age of the patients was 43 years (IQR 32–52), with 14 (6%) patients <18 years of age and 6 (3%) ≥80 years old; 122 (56%) were male. A total of 100 patients (46%) had known exposure to COVID-19, and 111 patients (51%) had recently traveled to Wuhan, China. There were four (2%) patients who had neither traveled recently to Wuhan nor had known exposure to confirmed COVID-19 patients who were nevertheless diagnosed with COVID-19, and the route of transmission in these cases might have been the use of public transportation. As for their personal medical history, 23 (11%) patients had a history of smoking, 38 (17%) had cardiovascular disease, 27 (12%) had diabetes, 14 (6%) had chronic pulmonary disease, 13 (6%) had liver disease, 10 (5%) had nutritional deficiency diseases, six (3%) had cerebrovascular disease, four (2%) had chronic renal diseases, two (1%) had cancer, and two (1%) had autoimmune diseases.

Disease Course

The patients' COVID-19 onset symptoms are shown in **Table 2**. Common clinical features included cough (162/218, 74%), fever (145/218, 67%), sputum production (99/218, 45%), and fatigue (77/218, 35%). Only 3% (6/218) of patients had nasal congestion and rhinorrhea. On admission, 39% (86/218) of patients had a recorded temperature of ≥38.1°C. No lung lesions were identified in the computed tomography (CT) scans of asymptomatic and mild cases. In moderate cases, the main imaging changes were ground-glass opacities and local patchy shadowing. In severe cases, the principal abnormality visible on CT scans was diffuse patchy shadowing. In critical cases, pulmonary consolidation and diffuse patchy shadowing were more common (**Table 3**). Several of the characteristic chest CT features of COVID-19 observed in the moderate, severe, and critical cases are shown in **Figure 2**. Although there was a notable degree of variability in the pattern of the infiltrates (ground-glass, local, diffuse, pulmonary consolidation), most patients had ground-glass opacities.

Laboratory Indices

Laboratory indices on admission are shown in **Table 4**. With increasing grades of disease severity based on clinical classification, the proportion of lymphocytes gradually decreased ($p = 0.001$). Elevated D-dimer levels were significantly associated with disease severity ($p < 0.000$), with high D-dimer levels in the severe ($0.76 \pm 1.22\,\mu g/mL$) and critical ($1.76 \pm 3.34\,\mu g/mL$) groups. With increasing grades of disease severity, the level of lactate dehydrogenase gradually increased ($p = 0.000$).

Treatment

The chief method of patient management was through symptomatic treatment. Regardless of severity, the vast majority of patients received antiviral treatment. Several patients had bacterial infections and were also given antibiotics. In detail, among the 218 patients, 192 (88%) patients received

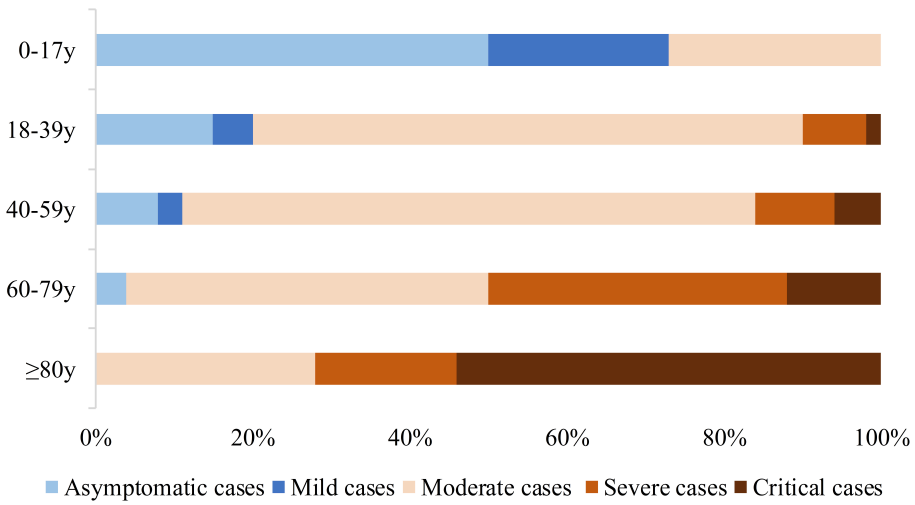


FIGURE 1 | Clinical classification (including asymptomatic cases) and age distribution of patients with COVID-19.

TABLE 2 | Symptoms (at the time of admission), comorbidities, treatments, and prognosis of patients with COVID-19.

	All patients ($n = 218$)	Asymptomatic cases ($n = 24$)	Mild cases ($n = 10$)	Moderate cases ($n = 146$)	Severe cases ($n = 24$)	Critical cases ($n = 14$)	*P*-value
Symptoms†							
Fever	145 (67%)	0	2 (20%)	108 (74%)	23 (96%)	12 (86%)	0.000**
<37.3°C	73 (33%)	24 (100%)	8 (80%)	38 (26%)	1 (4%)	2 (14%)	
37.3–38.0°C	59 (27%)	0	2 (20%)	45 (31%)	8 (33%)	4 (29%)	
38.1–39°C	70 (32%)	0	0	55 (38%)	11 (46%)	4 (29%)	
>39°C	16 (7%)	0	0	8 (5%)	4 (17%)	4 (29%)	
Cough	162 (74%)	0	10 (100%)	117 (80%)	21 (88%)	14 (100%)	0.000**
Sputum production	99 (45%)	0	4 (40%)	68 (47%)	15 (63%)	12 (86%)	0.018**
Fatigue	77 (35%)	0	1 (10%)	55 (38%)	12 (50%)	9 (64%)	0.006**
Shortness of breath	42 (19%)	0	0	16 (11%)	16 (67%)	10 (71%)	0.000**
Myalgia	41 (19%)	0	0	32 (22%)	6 (25%)	3 (21%)	0.407
Chills	39 (18%)	0	0	23 (16%)	9 (38%)	7 (50%)	0.001**
Headache	28 (13%)	0	1 (10%)	18 (12%)	4 (17%)	5 (36%)	0.125
Sore throat	25 (11%)	0	1 (10%)	20 (14%)	2 (8%)	2 (14%)	0.942
Diarrhea	16 (7%)	0	1 (10%)	11 (8%)	3 (13%)	1 (7%)	0.722
Nasal congestion and rhinorrhea	6 (3%)	0	0	6 (4%)	0	0	1.000
Complications							
ARDS	14 (6%)	0	0	0	0	14 (100%)	0.000**
Liver dysfunction	40 (18%)	0	1 (10%)	23 (16%)	9 (38%)	7 (50%)	0.000**
Acute kidney injury	10 (5%)	0	0	4 (3%)	1 (4%)	5 (36%)	0.001**
Acquired pneumonia	20 (9%)	0	0	3 (2%)	4 (17%)	13 (93%)	0.000**
Septic shock	4 (2%)	0	0	0	0	4 (29%)	0.000**
Treatment							
Oxygen treatment‡	156 (72%)	0	3 (30%)	115 (79%)	24 (100%)	14 (100%)	0.000**
Mechanical ventilation	16 (7%)	0	0	0	2 (8%)	14 (100%)	0.000**
Non-invasive	9 (4%)	0	0	0	2 (8%)	7 (50%)	
Invasive	7 (3%)	0	0	0	0	7 (50%)	
Prone position ventilation	14 (6%)	0	0	0	0	14 (100%)	0.000**
Renal replacement therapy	5 (2%)	0	0		11 (46%)	3 (21%)	0.000**
Convalescent plasma	4 (2%)	0	0	0	0	4 (17%)	0.000**
Stem cell treatment	3 (1%)	0	0	0	0	3 (21%)	0.000**
Lopinavir/ritonavir	192 (88%)	19 (79%)	7 (70%)	133 (91%)	20 (83%)	13 (93%)	0.172
Interferon alpha inhalation	192 (88%)	18 (75%)	7 (70%)	131 (90%)	23 (96%)	13 (93%)	0.059
Arbidol	126 (58%)	9 (38%)	3 (30%)	83 (57%)	18 (75%)	13 (93%)	0.001**
Antibiotics	115 (53%)	6 (25%)	1 (10%)	71 (49%)	23 (96%)	14 (100%)	0.000**
Chinese medicine§	196 (90%)	21 (88%)	9 (90%)	133 (91%)	20 (83%)	13 (93%)	0.714
Qingfei Paidu decoction	114 (52%)	17 (71%)	6 (60%)	75 (51%)	9 (38%)	7 (50%)	0.220
Lianhuaqingwen capsule	66 (30%)	3 (13%)	2 (20%)	47 (32%)	10 (42%)	4 (29%)	0.203
Huoxiangzhengqi liquid	6 (3%)	0	0	6 (4%)	0	0	0.822
Xuebijing injection	26 (12%)	0	0	11 (8%)	7 (29%)	8 (33%)	0.000**
Corticosteroid	47 (22%)	0	0	17 (12%)	18 (75%)	12 (86%)	0.000**
Gamma globulin	33 (15%)	0	0	13 (9%)	11 (46%)	9 (64%)	0.000**

(Continued)

TABLE 2 | Continued

	All patients ($n = 218$)	Asymptomatic cases ($n = 24$)	Mild cases ($n = 10$)	Moderate cases ($n = 146$)	Severe cases ($n = 24$)	Critical cases ($n = 14$)	*P*-value
Prognosis							
Discharge from hospital	217 (99.5%)	24 (100%)	10 (100%)	146 (100%)	24 (100%)	13 (93%)	0.000**
Death	1 (0.5%)	0	0	0	0	1 (7%)	
The hospitalization days of discharged patients	12.2 ± 6.2	7.1 ± 2.8[c,d,e]	8.6 ± 5.0[d,e]	12.1 ± 5.8[a,d,e]	16.1 ± 5.5[a,b,c,e]	20.5 ± 6.0[a,b,c,d]	0.000*

Data are mean (SD) or n (%).

**ANOVA was used for group comparisons with LSD for post-hoc tests.*

[a]vs. Asymptomatic cases ($p < 0.05$), [b]vs. Mild cases ($p < 0.05$), [c]vs. Moderate cases ($p < 0.05$), [d]vs. Severe cases ($p < 0.05$), [e]vs. Critical cases ($p < 0.05$).

***Statistical analysis was performed with the chi-square test or Fisher's exact test.*

[†]In part of this series, asymptomatic cases were not included in the statistics.

[‡]Oxygen therapy includes nasal catheter, mask oxygenation and nasal high-flow oxygen therapy.

[§]A small number of patients were given two or more kind of Chinese medicine.

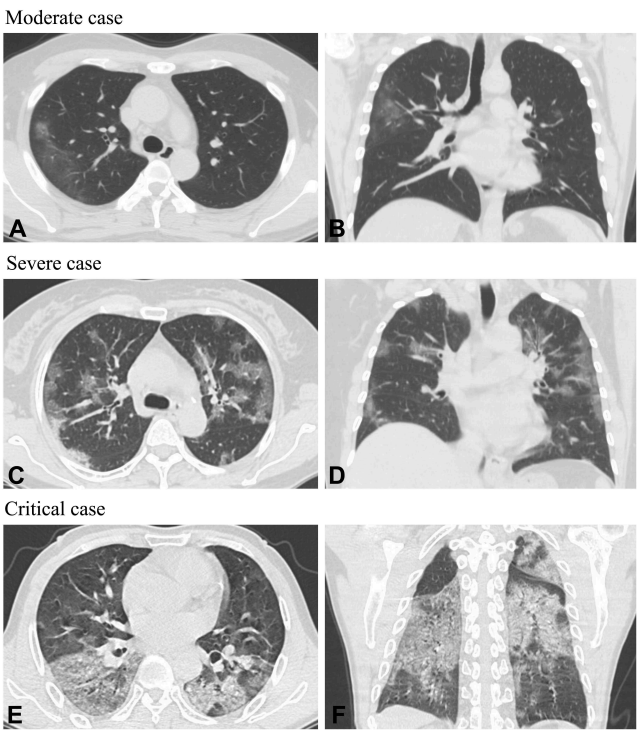


FIGURE 2 | Axial planes and coronal chest CT scans in patients with COVID-19. Moderate case: **(A,B)** Chest CT images of a 41-year-old man showed a ground-glass lesion in the right lobe on the 3rd day following a fever. Severe case: **(C,D)** Chest CT images of a 55-year-old woman showed bilateral multifocal ground-glass opacities on the 8th day after having chills, cough, and expectoration. Critical case: **(E,F)** Chest CT images of a 61-year-old man showed diffuse patchy shadowing and mixed consolidation on the 13th day after having cough, expectoration, and fever.

lopinavir/ritonavir, 192 (88%) patients received interferon-alpha inhalation, 126 (58%) patients received arbidol, 115 (53%) patients received antibiotics, 47 (22%) patients received corticosteroids, 33 (15%) patients received gamma globulin, four (2%) patients received convalescent plasma, and three (1%) patients received umbilical cord mesenchymal stem cell treatment. For respiratory support, 156 (72%) patients were treated with oxygen treatment (including a nasal catheter, mask oxygenation, and/or nasal high-flow oxygen therapy), 16 (7%) with mechanical ventilation, and 14 (6%) with prone position ventilation. Five (2%) patients required renal replacement therapy. Most distinctive is that the majority of cases (196/218, 90%) received traditional Chinese medicine, which is a different treatment approach from that used in other countries. Among these Chinese medicines, the Qingfei Paidu decoction (114/218, 52%) and Lianhuaqingwen capsules (66/218, 30%) were the most frequently used. Huoxiangzhengqi liquid was used only in patients with gastrointestinal discomfort, while the Xuebijing injection was mainly used for severe and critical patients (**Table 2**).

Prognosis

There was one death in our cohort of 218 hospitalized COVID-19 patients. This patient had diabetes, hypertension, and severe obesity. As of March 14, most individuals (217/218 [99.5%]) had recovered and were discharged from the hospital. Among the patients who survived, the median hospital stay was 12.2 days (IQR 8–16 days). There were 25 (11%) patients who developed serious conditions during hospitalization, including pulmonary aggravation requiring oxygen ventilation or transfer to an ICU, and 13 patients did not receive steroids during the early stage of the disease but were treated with corticosteroids at a later stage. Nine (<1%) patients had rapid disease progression.

Among the whole cohort, 11% of patients (25/218) were admitted to the ICU and 7% (16/218) received mechanical ventilation. Of the 6% of patients (14/218) diagnosed with ARDS, all belonged to the critical group of cases. Among the 16 patients who received mechanical ventilation, one (6%) died, and the remaining 15 (94%) were discharged before March 14, 2020. Overall, 25 patients in our cohort met the criteria for a poor outcome (death or ICU admission with or without mechanical ventilation). The majority of these poor outcomes occurred within 10 days of hospitalization.

Table 5 shows summaries of the age, sex, clinical classification, and initial laboratory results of patients classified as having a

TABLE 3 | Chest CT/X-ray features of patients with COVID-19 at the most severe stage.

Distribution of pulmonary lesions	All patients ($n = 218$)	Asymptomatic cases ($n = 24$)	Mild cases ($n = 10$)	Moderate cases ($n = 146$)	Severe cases ($n = 24$)	Critical cases ($n = 14$)
No lesion	37 (17%)	24 (100%)	10 (100%)	3 (2%)	0	0
Ground-glass opacities	65 (30%)	0	0	65 (45%)	0	0
Local patchy shadowing	77 (35%)	0	0	76 (52%)	1 (4%)	0
Diffuse patchy shadowing	30 (14%)	0	0	2 (1%)	21 (88%)	7 (50%)
Pulmonary consolidation	9 (4%)	0	0	0	2 (8%)	7 (50%)

A total of 205- chest CT cases included in this table. Other 13 patients had chest X-ray.

TABLE 4 | Initial laboratory results of patients with COVID-19.

	All patients ($n = 218$)	Asymptomatic cases ($n = 24$)	Mild cases ($n = 10$)	Moderate cases ($n = 146$)	Severe cases ($n = 24$)	Critical cases ($n = 14$)	*P*-value
Hematologic							
Leucocytes ($\times 10^9$/L; reference range 3.69–9.16)	5.92 ± 3.23	6.22 ± 2.06	5.19 ± 1.40	5.72 ± 3.18	6.39 ± 3.89	7.24 ± 4.30	0.407
Lymphocytes ($\times 10^9$/L; reference range 0.8–4.0)	1.25 ± 0.61	1.68 ± 0.79^{d,e}	1.95 ± 0.67^{d,e}	1.26 ± 0.55^{d,e}	0.90 ± 0.40^{a,b,c}	0.76 ± 0.33^{a,b,c}	0.001*
Coagulation function							
APTT (s; reference range 23.0–40.0)	33.64 ± 13.51	36.49 ± 13.70^{e}	34.86 ± 7.76^{e}	31.64 ± 7.45^{e}	32.54 ± 4.70^{e}	50.37 ± 38.1^{a,b,c,d}	0.001*
D-dimer (μg/ml; reference range 0.0–0.7)	0.45 ± 1.06	0.31 ± 0.18^{e}	0.212 ± 0.083^{e}	0.29 ± 0.22^{e}	0.76 ± 1.22^{e}	1.76 ± 3.34^{a,b,c,d}	0.000*
Biochemistry							
Alanine aminotransferase (U/L; reference range 0–40.0)	27.88 ± 21.62	23.54 ± 16.75	18.99 ± 6.92	27.62 ± 20.16	36.90 ± 34.88	24.76 ± 11.76	0.173
Aspartate aminotransferase (U/L; reference range 0–40.0)	27.75 ± 13.55	19.36 ± 7.77^{c,d,e}	22.80 ± 6.58^{d}	27.37 ± 13.11^{a,d}	34.08 ± 17.86^{a,b,c}	33.44 ± 11.23^{a}	0.004*
Serum creatinine (μmol/L; reference range 53.0–115·0)	72.39 ± 56.64	65.81 ± 21.27	64.83 ± 10.07	67.47 ± 37.33	102.47 ± 137.40	84.66 ± 21.84	0.082
Serum urea (mmol/L; reference range 2.86–7.14)	4.21 ± 3.02	3.89 ± 1.39	3.79 ± 0.48	4.04 ± 3.40	4.95 ± 2.09	5.27 ± 1.75	0.440
Lactate dehydrogenase (U/L; reference range 114.0–240.0)	236.77 ± 216.84	167.47 ± 47.54^{e}	172.71 ± 41.18^{e}	212.07 ± 76.62^{e}	292.90 ± 85.76^{e}	510.06 ± 733.24^{a,b,c,d}	0.000*
C-reactive protein (mg/L; reference range 0–3.0)	18.57 ± 33.82	1.64 ± 2.34^{d,e}	0.95 ± 0.73^{d,e}	13.46 ± 23.58^{d,e}	38.70 ± 51.53^{a,b,c,e}	66.01 ± 54.34^{a,b,c,d}	0.000*
Erythrocyte sedimentation rate (mm/h; reference range 0–20.0)	41.09 ± 31.72	14.00 ± 19.76^{c,d,e}	14.60 ± 21.9^{d,e}	43.03 ± 30.24^{a}	50.56 ± 31.69^{a,b}	61.00 ± 37.98^{a,b}	0.000*
PaO2/FiO2 (mm Hg; reference range 400–500)†	NA	NA	NA	NA	NA	176 ± 49	..

Data are n (%), n/N (%), mean (SD), and median (IQR).

**ANOVA was used for group comparisons with LSD for post-hoc tests.*

^{a}vs. Asymptomatic cases ($p < 0.05$), ^{b}vs. Mild cases ($p < 0.05$), ^{c}vs. Moderate cases ($p < 0.05$), ^{d}vs. Severe cases ($p < 0.05$), ^{e}vs. Critical cases ($p < 0.05$).

†We analyzed the data when patients were monitored in ICU.

poor prognosis. Univariate analysis of these data showed that advanced age, disease severity (based on clinical classification), an increased activated partial thromboplastin time (APTT), a higher erythrocyte sedimentation rate, and elevated levels of lactate dehydrogenase and C-reaction protein were significantly associated with poor outcome. Lymphopenia was also significantly associated with poor outcome.

Following univariate analysis (**Table 5**), the Cox proportional hazards model showed that the risk of a poor outcome was increased for those aged 60 years or older [relative risk (RR), 3.6; 95% CI, 1.6–8.0; $p = 0.001$]. The presence of any comorbid disease (other than diabetes) was found to increase the risk of a poor outcome (RR, 8.9; 95% CI, 3.0–26.0; $p = 0.000$), as was the presence of diabetes (RR, 5.9; 95% CI, 2.7–13.0; $p = 0.000$).

TABLE 5 | Analysis of poor outcome and clinical features.

Variable	Univariate analysis, mean (IQR)		
	No poor outcome ($n = 193$)	**Poor outcome[†] ($n = 25$)**	***P*-value**
Age, y	40.9 (30.0–50.0)	58.4 (49.0–67.5)	0.000
Men, %	107 (55%)	15 (60%)	0.831
Clinical classification			0.000
Critical cases	0	14 (56%)	..
Severe cases	13 (7%)	11 (44%)	..
Moderate cases	146 (76%)	0	..
Mild cases	10 (5%)	0	..
Asymptomatic cases	24 (12%)	0	..
Leucocytes, $\times 10^9$/L	5.87 ± 3.19	6.34 ± 3.50	0.493
Lymphocytes, $\times 10^9$/L	1.31 ± 0.61	0.80 ± 0.34	0.000
APTT, s	23.89 ± 16.71	39.17 ± 33.99	0.045
ALT, U/L	27.81 ± 22.57	28.40 ± 13.37	0.899
AST, U/L	27.12 ± 13.78	32.22 ± 11.02	0.078
Scr, μmol/L	71.78 ± 59.78	77.00 ± 21.26	0.679
LDH, U/L	211.08 ± 77.18	433.43 ± 574.54	0.000
CRP, mg/L	12.89 ± 24.69	58.84 ± 56.48	0.000
ESR, mm/h	38.51 ± 30.58	62.43 ± 34.03	0.001
	Univariate analysis[‡] Relative risk (95% CI) of poor outcome[§]		***P*-value**
Age≥60 y	3.6 (1.6–8.0)		0.001
Diabetes	5.9 (2.7–13.0)		0.000
Other comorbid disease	8.9 (3.0–26.0)		0.000
	Multivariable analysis[‡] Relative risk (95% CI) of poor outcome[§]		***P*-value**
Age≥60 y	1.9 (0.8–4.2)		0.134
Diabetes	3.0 (1.3–6.8)		0.007
Other comorbid disease	5.9 (1.9–17.8)		0.002

Data are median (IQR), n (%), or mean (SD).

APTT, Activated partial thromboplastin time; ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; Scr, Serum creatinine; LDH, Lactate dehydrogenase; CRP, C-reaction protein; ESR, Erythrocyte sedimentation rate.

[†] Defined as death or intensive care unit admission with or without mechanical ventilation.

[‡] Results are from Cox proportional hazards model.

[§] Reference group is younger than 60 years, with no diabetes, and no other comorbid disease (chronic pulmonary disease, cardiovascular disease, chronic renal diseases, cerebrovascular disease, liver disease, cancer, malnutrition, or autoimmune disease).

Multivariable Cox proportional hazards analysis was performed with the *a priori* hypothesis that age and comorbid diseases were independently associated with a poor outcome (**Table 5**). In the model including diabetes, other comorbid diseases, and an age ≥60 years, no significant association was found between advanced age and poor outcome (RR, 1.9; 95% CI, 0.8–4.2; $p = 0.134$). However, diabetes alone or with other diseases (RR, 3.0; 95% CI, 1.3–6.8; $p = 0.007$) and any comorbid diseases other than diabetes (cardiovascular disease, chronic pulmonary disease, and other chronic diseases; RR, 5.9; 95% CI, 1.9–17.8; $p = 0.002$) were independently associated with a poor outcome.

Despite age ≥60 years, diabetes, and other chronic diseases all being positively associated with a poor outcome, a comparison of the parameter estimates as well as the standard errors in the single and multivariable models indicated that collinearity was not apparent. The standard error for the age parameter was only marginally larger in the multivariable models than in the univariate regression model of age alone. **Figure 3** shows the Kaplan–Meier survival curves for these three groups defined by the presence and absence of diabetes and other chronic comorbidities.

Follow-Up

To date, 20 patients (20/218, 9%) have retested positive for SARS-CoV-2 RNA in nasopharyngeal swabs after having recovered and being discharged. Among these, 18 were classified into the moderate disease group and two were classified into the mild group upon their first admission. These patients showed

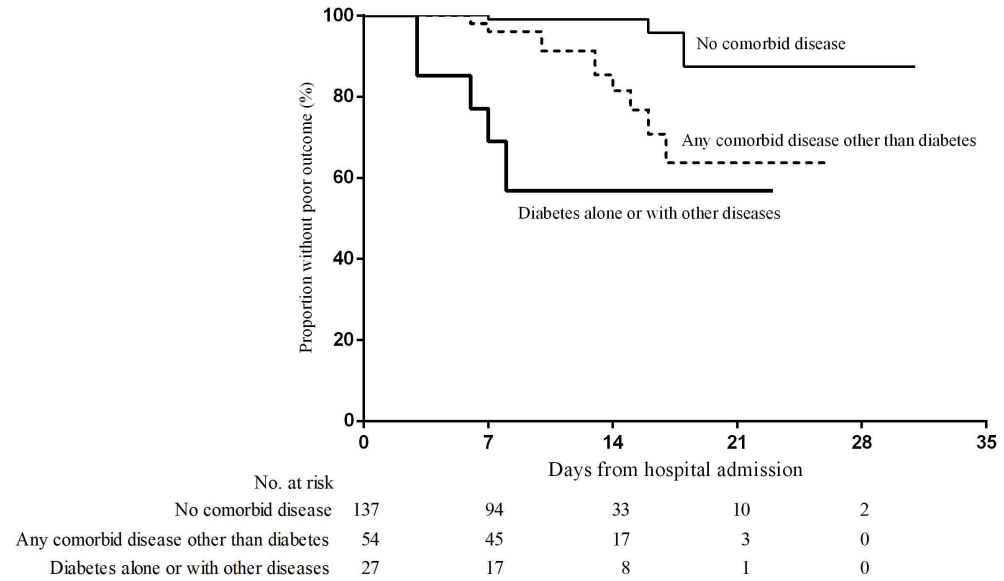


FIGURE 3 | Time from admission to poor outcome based on the presence of a comorbid disease. Poor outcome was defined as death or intensive care unit admission with or without mechanical ventilation.

relatively mild symptoms or were asymptomatic during follow-up. Thus, far, no critical or severe cases have retested positive after being discharged.

DISCUSSION

Here, the characteristics of a cohort of 218 COVID-19 patients were summarized based on clinical classification of disease severity. This study reflects China's initial experience as the first country to respond to the virus. These findings have important clinical, infection control, and public health implications.

Most patients were clinically classified as moderate cases and had a good prognosis. The median age of the patients increased with the clinical classification of disease severity. Continued vigilance is, therefore, warranted for this high-risk group. The prognosis for the elderly and patients with diabetes and other chronic comorbidities was poor. We attempted to analyze the role of each comorbid disease in COVID-19; however, the number of patients was too small to perform statistical analyses when subgrouping each comorbid disease separately. As for diabetes, previous studies have shown that diabetes can affect the prognosis of patients with viral pneumonia and it should, therefore, be analyzed separately from other comorbid diseases (15). In the univariate analysis performed here and in a previous study by Chen et al. (17), diabetes was found to be associated with poor COVID-19 outcome. For this reason, we analyzed data from the diabetes patients separately from those with other comorbid diseases.

The hallmark laboratory findings of our study indicated that elevated levels of lactate dehydrogenase, C-reaction protein, and D-dimer, as well as an increased erythrocyte sedimentation rate, were positively correlated with clinical classification. Thus, these factors may be involved in disease progression and should receive further attention.

Asymptomatic cases comprised 11% of our cohort, suggesting that there may be a large number of asymptomatic patients in the general population who have not been tested and are transmitting the virus (18). In agreement with a report by Guan et al. who studied a cohort of 1,099 COVID-19 patients in China (6), the most common symptoms reported here were cough, fever, sputum production, and fatigue. Cough was the first symptom reported by many patients (74%). Only 3% of patients had nasal congestion and rhinorrhea, which may assist in differentiating this disease from the common cold.

Most patients had positive CT images. CT imaging has been observed to show multiple ground-glass opacities and even infiltration in both lungs as COVID-19 progresses (19, 20). In severe cases, pulmonary consolidation may be found (19). Chest CT is very important for COVID-19 diagnosis and patient management. Therefore, if medical conditions permit, it is recommended that patients undergo follow-up CT (20).

Currently, no standard treatment has been recommended for coronavirus infection besides careful supportive care (11, 21–23). Given the retrospective nature of our study, it was difficult to determine whether there was any therapeutic benefit conferred by the treatment regimens used for COVID-19, particularly the antibiotic and corticosteroid treatments (24). Treatment with lopinavir/ritonavir was previously reported to show potential in the treatment of SARS, and it can be supposed that this treatment may be beneficial in the treatment of COVID-19 (25).

Recent reports suggest that patients recover from COVID-19 when they receive combined traditional Chinese and Western medicine (23). In our cohort, 53% of patients received antibacterial agents, 88% received antiviral therapy, and 22% received methylprednisolone. Furthermore, 90% received Chinese medicine treatment. The favorable outcome observed for most cases in this cohort may support a COVID-19 treatment approach comprising a combination of traditional Chinese

medicine and modern therapies (26). Notably, the most common Chinese medicines, the Lianhuaqingwen capsule and Qingfei Paidu decoction, have proven to be effective in viral pneumonia (27, 28), whereas the Xuebijing injection has been used for severe pneumonia for many years (29).

In agreement with Guan et al. (6), only 7% of the patients in our cohort required mechanical ventilation. Furthermore, we observed a low crude mortality rate (0.5%). This may be related to early nucleic acid detection in close contacts, as well as the relatively low incidence and adequate medical resources found in Hunan province (2). Cases with an exposure history tended to have a milder clinical classification, which may be owing to the vigilance of patients and healthcare workers in seeking early diagnosis and treatment.

Age, lymphocytes, lactate dehydrogenase, C-reaction protein, and erythrocyte sedimentation rate were all associated with the clinical classification. In our multivariable Cox proportional hazards model, diabetes and other chronic comorbid conditions were independently associated with poor prognosis, although an age of 60 years and older was not. Larger sample studies are needed to further elucidate which patients are at most risk of death or requiring admission to an ICU (8).

Currently, the RT-PCR is the standard test for the diagnosis of COVID-19 (11, 30). Notably, the infection appears to be transmitted during the incubation period of the index patient, in whom the illness is brief and non-specific (31). Asymptomatic cases in this study comprised 11% of the patients, all of whom were potential sources of SARS-CoV-2 infection (32, 33). To increase the positive rate of nucleic acid testing, we recommend that sputum and nasopharyngeal swabs be retained as much as possible (11). We further recommend that RT-PCR be repeated twice or more for suspected cases and close contacts as early as possible. This can facilitate early diagnosis, early isolation, and early treatment, and help to reduce the spread of disease (34).

The main strength of our study lies in the application of a new method for clinical classification. Zhang et al. studied the clinical and laboratory characteristics of 140 community-infected COVID-19 patients (35). They compared the data between only severe and non-severe groups, which were defined according to clinical severity. Here, the clinical classification of COVID-19 was performed by referencing the *Diagnosis and treatment protocol for COVID-19 (trial version 7)* (11), which is the latest version of the clinical practice guidelines and has stricter criteria. In this way, the classification and category distribution of groups were described comprehensively and systematically. Using this approach, we found that the moderate cases were the most common. In contrast, the proportions of severe and critical cases were relatively small. In the context of the high prevalence of SARS-CoV-2, the current clinical classification is particularly significant for the guidance of patient management and treatment. Further, our pilot results showed that most of the patients who retested positive for SARS-CoV-2 were from the moderate and mild groups. As such, our classification approach may have implications for clinical monitoring, treatment, and prognosis.

Our study had several limitations. One was the relatively low number of patients and critical cases included. A larger sample size with a greater proportion of critical cases is necessary for future investigations. Moreover, our study was not a randomized controlled trial but rather a retrospective study. Multiple drugs were used, making it difficult to evaluate the effectiveness of a single treatment. Hence, randomized, controlled, multicenter clinical trials are needed to confirm the present findings. As a retrospective observation, the main focus of this study was the nucleic acids present in swabs from the respiratory system. Testing stool nucleic acid is a valuable complementary tool to better understand COVID-19 progression and transmission. Future projects investigating the clinical longitudinal changes in COVID-19 should take the stool nucleic acid test into consideration.

In conclusion, despite the widespread implications of COVID-19, most patients have a favorable clinical prognosis. The COVID-19 epidemic has placed enormous strain on the health and economic status of nations. The excellent spirit of international collaboration among clinicians, researchers, and government agencies needs to continue in an effort to better control and treat COVID-19 (36–38).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of Shaoyang Central Hospital; Ethics Committee of Loudi Central Hospital; Ethics Committee of Xiangtan Central Hospital. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

XY and YZ contributed to the study concept and design of this study. XY, XH, DP, YF, ZF, DL, YX, SZ, FC, and WL contributed to the acquisition, analysis, interpretation of data, and the drafting of the paper. YZ contributed to the review and the revision of the manuscript. All authors give final approval to this manuscript for publication.

FUNDING

This study was funded by the Program of Hunan Science and Technology Department (2020SK3011).

ACKNOWLEDGMENTS

We thank all patients involved in the study.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2020.00485/full#supplementary-material

REFERENCES

1. Zhu N, Zhang D, Wang W, Li X, Yang B, Song J, et al. A novel coronavirus from patients with pneumonia in China, 2019. *N Engl J Med.* (2020) 382:727–33. doi: 10.1056/NEJMoa2001017
2. WHO. *Coronavirus Disease (COVID-2019) Situation Reports*. Available online at: https://www.who.int/emergencies/diseases/novel-coronavirus-2019/situation-reports (accessed April 18, 2020)
3. Thompson R. Pandemic potential of 2019-nCoV. *Lancet Infect Dis.* (2020) 20:280. doi: 10.1016/S1473-3099(20)30068-2
4. Wang C, Horby PW, Hayden FG, Gao GF. A novel coronavirus outbreak of global health concern. *Lancet.* (2020) 395:470–3. doi: 10.1016/S0140-6736(20)30185-9
5. Chen N, Zhou M, Dong X, Qu J, Gong F, Han Y, et al. Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study. *Lancet.* (2020) 395:507–13. doi: 10.1016/S0140-6736(20)30211-7
6. Guan WJ, Ni ZY, Hu Y, Liang WH, Ou CQ, He JX, et al. Clinical characteristics of coronavirus disease 2019 in China. *N Engl J Med.* (2020) 382:1708–20. doi: 10.1056/NEJMoa2002032
7. Wang D, Hu B, Hu C, Zhu F, Liu X, J. Zhang, et al. Clinical characteristics of 138 hospitalized patients with 2019 novel coronavirus-infected pneumonia in Wuhan, China. *JAMA*. (2020) 323:1061–9. doi: 10.1001/jama.2020.1585
8. Yang X, Yu Y, Xu J, Shu H, Xia J, Liu H, et al. Clinical course and outcomes of critically ill patients with SARS-CoV-2 pneumonia in Wuhan, China: a single-centered, retrospective, observational study. *Lancet Respir Med.* (2020) 8:475–81. doi: 10.1016/S2213-2600(20)30079-5
9. Xu Z, Shi L, Wang Y, Zhang J, Huang LC, Zhang, et al. Pathological findings of COVID-19 associated with acute respiratory distress syndrome. *Lancet Respir Med.* (2020) 8:420–2. doi: 10.1016/S2213-2600(20)30076-X
10. Wu C, Chen X, Cai Y, Xia J, Zhou X, S. Xu, et al. Risk factors associated with acute respiratory distress syndrome and death in patients with coronavirus disease 2019 pneumonia in Wuhan, China. *JAMA Intern Med*. (2020) 180:1–11. doi: 10.1001/jamainternmed.2020.0994
11. National Health Commission of the People's Republic of China. *Diagnosis and Treatment Protocol for COVID-19 (trial version 7)*. Available online at: http://www.nhc.gov.cn/xcs/zhengcwj/202003/46c9294a7dfe4cef80dc7f5912eb1989.shtml (in Chinese) (accessed March 3, 2020).
12. Pan X, Chen D, Xia Y, Wu X, Li T, Ou X, et al. Asymptomatic cases in a family cluster with SARS-CoV-2 infection. *Lancet Infect Dis.* (2020) 20:410–1. doi: 10.1016/S1473-3099(20)30114-6
13. Zhang J, Tian S, Lou J, Chen Y. Familial cluster of COVID-19 infection from an asymptomatic. *Crit Care.* (2020) 24:119. doi: 10.1186/s13054-020-2817-7
14. Ranieri VM, Rubenfeld GD, Thompson BT, Ferguson ND, Caldwell E, Fan E, et al. Acute respiratory distress syndrome: the Berlin definition. *JAMA*. (2012) 307:2526–33. doi: 10.1001/jama.2012.5669
15. Booth CM, Matukas LM, Tomlinson GA, Rachlis AR, Rose DB, Dwosh HA, et al. Clinical features and short-term outcomes of 144 patients with SARS in the greater Toronto area. *JAMA*. (2003) 289:2801–9. doi: 10.1001/jama.289.21.JOC30885
16. Gomersall CD, Joynt GM, Lam P, Li T, Yap F, Lam D, et al. Short-term outcome of critically ill patients with severe acute respiratory syndrome. *Intensive Care Med.* (2004) 30:381–7. doi: 10.1007/s00134-003-2143-y
17. Chen Y, Yang D, Cheng B, Chen J, Peng A, Yang C, et al. Clinical characteristics and outcomes of patients with diabetes and COVID-19 in association with glucose-lowering medication. *Diabetes Care.* (2020) 43:1399–407. doi: 10.2337/dc20-0660
18. Yan X, Han X, Fan Y, Fang Z, Long D, Zhu Y. Duration of SARS-CoV-2 viral RNA in asymptomatic carriers. *Crit Care.* (2020) 24:245. doi: 10.1186/s13054-020-02952-0
19. Shi H, Han X, Jiang N, Cao Y, Alwalid O, Gu J, et al. Radiological findings from 81 patients with COVID-19 pneumonia in Wuhan, China: a descriptive study. *Lancet Infect Dis.* (2020) 20:425–34. doi: 10.1016/S1473-3099(20)30086-4
20. Lee EYP, Ng MY, Khong PL. COVID-19 pneumonia: what has CT taught us? *Lancet Infect Dis.* (2020) 20:384–5. doi: 10.1016/S1473-3099(20)30134-1
21. Chen L, Xiong J, Bao L, Shi Y. Convalescent plasma as a potential therapy for COVID-19. *Lancet Infect Dis.* (2020) 20:398–400. doi: 10.1016/S1473-3099(20)30141-9
22. Stebbing J, Phelan A, Griffin I, Tucker C, Oechsle O, Smith D, et al. COVID-19: combining antiviral and anti-inflammatory treatments. *Lancet Infect Dis.* (2020) 20:400–2. doi: 10.1016/S1473-3099(20)30132-8
23. Wang Z, Chen X, Lu Y, Chen F, Zhang W. Clinical characteristics and therapeutic procedure for four cases with 2019 novel coronavirus pneumonia receiving combined Chinese and Western medicine treatment. *Biosci Trends.* (2020) 14:64–8. doi: 10.5582/bst.2020.01030
24. Russell CD, Millar JE, Baillie JK. Clinical evidence does not support corticosteroid treatment for 2019-nCoV lung injury. *Lancet.* (2020) 395:473–5. doi: 10.1016/S0140-6736(20)30317-2
25. Chu CM, Cheng VC, Hung IF, Wong MM, Chan KH, Chan KS, et al. Role of lopinavir/ritonavir in the treatment of SARS: initial virological and clinical findings. *Thorax.* (2004) 59:252–6. doi: 10.1136/thorax.2003.012658
26. Zhang DH, Wu KL, Zhang X, Deng SQ, Peng B. In silico screening of Chinese herbal medicines with the potential to directly inhibit 2019 novel coronavirus. *J Integr Med.* (2020) 18:152–8. doi: 10.1016/j.joim.2020.02.005
27. Runfeng L, Yunlong H, Jicheng H, Weiqi P, Qinhai M, Yongxia S, et al. Lianhuaqingwen exerts anti-viral and anti-inflammatory activity against novel coronavirus (SARS-CoV-2). *Pharmacol Res.* (2020) 156:104761. doi: 10.1016/j.phrs.2020.104761
28. Luo E, Zhang D, Luo H, Liu B, Zhao K, Zhao Y, et al. Treatment efficacy analysis of traditional Chinese medicine for novel coronavirus pneumonia (COVID-19): an empirical study from Wuhan, Hubei Province, China. *Chin Med.* (2020) 15:34. doi: 10.1186/s13020-020-00317-x
29. Song Y, Yao C, Yao Y, Han H, Zhao X, Yu K, et al. XueBiJing injection versus placebo for critically Ill patients with severe community-acquired pneumonia: a randomized controlled trial. *Crit Care Med.* (2019) 47:e735–43. doi: 10.1097/CCM.0000000000003842
30. Corman VM, Landt O, Kaiser M, Molenkamp R, Meijer A, Chu DK, et al. Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. *Euro Surveill.* (2020) 25:2000045. doi: 10.2807/1560-7917.ES.2020.25.3.2000045
31. Chan JF, Yuan S, Kok KH, To KK, Chu H, Yang J, et al. A familial cluster of pneumonia associated with the 2019 novel coronavirus indicating person-to-person transmission: a study of a family cluster. *Lancet.* (2020) 395:514–23. doi: 10.1016/S0140-6736(20)30154-9
32. Rothe C, Schunk M, Sothmann P, Bretzel G, Froeschl G, Wallrauch C, et al. Transmission of 2019-nCoV infection from an asymptomatic contact in Germany. *N Engl J Med.* (2020) 382:970–1. doi: 10.1056/NEJMc2001468
33. Bai Y, Yao L, Wei T, Tian F, Jin DY, Chen L, et al. Presumed asymptomatic carrier transmission of COVID-19. *JAMA*. (2020) 323:1406–7. doi: 10.1001/jama.2020.2565
34. Hellewell J, Abbott S, Gimma A, Bosse NI, Jarvis CI, Russell TW, et al. Feasibility of controlling COVID-19 outbreaks by isolation of cases and contacts. *Lancet Glob Health.* (2020) 8:e488–96. doi: 10.1016/S2214-109X(20)30074-7
35. Zhang JJ, Dong X, Cao YY, Yuan YD, Yang YB, Yan YQ, et al. Clinical characteristics of 140 patients infected with SARS-CoV-2 in Wuhan, China. *Allergy*. (2020) 75:1730–41. doi: 10.1111/all.14238
36. Calisher C, Carroll D, Colwell R, Corley RB, Daszak P, Drosten C, et al. Statement in support of the scientists, public health professionals, and medical professionals of China combatting COVID-19. *Lancet.* (2020) 395:e42–3. doi: 10.1016/S0140-6736(20)30418-9
37. Xu Z, Li S, Tian S, Li H, Kong LQ. Full spectrum of COVID-19 severity still being depicted. *Lancet.* (2020) 395:947–8. doi: 10.1016/S0140-6736(20)30308-1
38. Heymann DL. Data sharing and outbreaks: best practice exemplified. *Lancet*. (2020) 395:469–70. doi: 10.1016/S0140-6736(20)30184-7

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Medicine

REVIEW
published: 13 August 2020
doi: 10.3389/fmed.2020.00526

Pulmonary and Extra-Pulmonary Clinical Manifestations of COVID-19

Kemmian D. Johnson[1], Christen Harris[1], John K. Cain[2], Cicily Hummer[3], Hemant Goyal[4] and Abhilash Perisetti[5]*

[1] Department of Internal Medicine, Louisiana State University Health Sciences Center, New Orleans, LA, United States, [2] Department of Nurse Anesthesia, Texas Christian University, Fort Worth, TX, United States, [3] College of Medicine, Rocky Vista University College of Osteopathic Medicine, Parker, CO, United States, [4] Department of Internal Medicine, The Wright Center for Graduate Medical Education, Scranton, PA, United States, [5] Department of Gastroenterology and Hepatology, University of Arkansas for Medical Sciences, Little Rock, AR, United States

The severe acute respiratory syndrome coronavirus−2 (SARS-CoV-2) has been recently identified as the culprit of the highly infectious, outbreak named coronavirus disease 2019 (COVID-19) in China. Now declared a public health emergency, this pandemic is present in more than 200 countries with over 14 million cases and 600,000 deaths as of July 18, 2020. Primarily transmitted through the respiratory tract, the most common clinical presentations of symptomatic individuals infected with SARS-CoV-2 include fever, dyspnea, cough, fatigue, and sore throat. In advanced cases, patients may rapidly develop respiratory failure with acute respiratory distress syndrome, and even progress to death. While it is known that COVID-19 manifests similarly to the 2003 Severe Acute Respiratory Syndrome (SARS) and the 2012 Middle East Respiratory Syndrome (MERS), primarily affecting the pulmonary system, the impact of the disease extends far beyond the respiratory system and affects other organs of the body. The literature regarding the extrapulmonary manifestations (cardiovascular, renal, hepatic, gastrointestinal, ocular, dermatologic, and neurological) of COVID-19 is scant. Herein, we provide a comprehensive review of the organ-specific clinical manifestations of COVID-19, to increase awareness about the various organs affected by SARS-CoV-2 and to provide a brief insight into the similarities and differences in the clinical manifestations of COVID-19 and the earlier SARS and MERS.

Keywords: COVID-19, SARS-CoV-2, coronavirus 2019, COVID-19, pneumonia, SARS-CoV-1, MERS-CoV

OPEN ACCESS

Edited by:
Hsiao-Chi Chuang,
Taipei Medical University, Taiwan

Reviewed by:
Nitya Singh,
University of Florida, United States
Ahmed Mostafa,
National Research Centre, Egypt

****Correspondence:***
Hemant Goyal
doc.hemant@yahoo.com

Specialty section:
This article was submitted to Infectious Diseases Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: *01 May 2020*
Accepted: *27 July 2020*
Published: *13 August 2020*

Citation:
Johnson KD, Harris C, Cain JK, Hummer C, Goyal H and Perisetti A (2020) Pulmonary and Extra-Pulmonary Clinical Manifestations of COVID-19. Front. Med. 7:526. doi: 10.3389/fmed.2020.00526

INTRODUCTION

Over the last two decades, the coronavirus family has been identified as the source of several highly pathogenic global outbreaks. Some of the most notable are the 2003 Severe Acute Respiratory Syndrome coronavirus-1 (SARS-CoV-1), which caused the Severe Acute Respiratory Syndrome (SARS) outbreak in China, and the 2012 Middle East Respiratory Syndrome coronavirus (MERS-CoV) which caused the MERS outbreak in Saudi Arabia (1, 2). The most recent coronavirus outbreak likely developed in a local market ("wet market") in China in December 2019 as a series of acute respiratory disorders [acute hypoxic respiratory failure, pneumonia, acute respiratory distress syndrome (ARDS) (3, 4)]. The causative pathogenic agent was found to be an enveloped, non-segmented, positive-sense RNA β-coronavirus, now termed Severe Acute

Respiratory Syndrome coronavirus-2 (SARS-CoV-2). The disease is referred to as the coronavirus disease 2019 or COVID-19 (3).

The most commonly affected organ system by COVID-19 is the pulmonary system, with the most frequent clinical manifestations including cough, dyspnea, fever, and sore throat, similar to SARS and MERS (5, 6). In the severe disease state, the patient's clinical course is complicated by the development of pneumonia with ARDS, acute hypoxic respiratory failure, and/or death (7). While the pulmonary system is most commonly affected, extrapulmonary organs and organ systems (including the cardiac, gastrointestinal, hepatic, renal, ocular, and dermatologic) are also affected by COVID-19, which could have significant health consequences.

COVID-19-related mortality has affected more individuals than its antecedents, SARS and MERS, combined. The number of identified cases is steadily growing, and the outbreak has rapidly spread to many different areas in China and more than 200 other countries in a short period of time (**Figure 1**). As of July 18, 2020, 14 million cases and 600,000 deaths have been documented globally across over 200 countries and territories (8). Therefore, understanding the clinical manifestations of COVID-9 is crucial.

In this article, we summarize the clinical manifestations of COVID-19 with an organ system-based approach to educate healthcare practitioners about both common and uncommon presentations of COVID-19 and to stay vigilant about this new disease (**Figure 2**).

PULMONARY SYSTEM

By far, the pulmonary system is the most common organ system affected by SARS-CoV-2. Several retrospective studies have consistently reported pulmonary manifestations in patients with COVID-19, which include cough, shortness of breath, sputum production, respiratory failure, and ARDS (**Table 1**) (5, 7, 9–17). In one large study ($n = 1{,}099$) from China, Guan et al. reported that 67.8% of patients with COVID-19 presented with cough, while 33% had sputum production, and 18.7% experienced shortness of breath (9). Similarly, another study ($n = 262$) of patients in Beijing demonstrated that cough occurred in almost half (45.8%) of patients with COVID-19, and dyspnea occurred in nearly 7% of patients (18). Multiple studies conducted in various countries have also demonstrated similar findings, showing that cough is the predominant pulmonary symptom in patients with COVID-19 (10, 19, 20). The main reason for the development of these symptoms is the presence of severe pneumonia in COVID-19 patients. However, the pulmonary symptoms can vary in COVID-19 patients, possibly due to variation in severity of disease at the time of presentation. In a study ($n = 41$) by Huang et al. on patients with confirmed SARS-CoV-2 infection, the most common symptoms were fever (98%) followed by cough (76%), with over half (55%) of the patients developing dyspnea (5).

ARDS is a known severe pulmonary complication of COVID-19, where patients experience severe hypoxia refractory to oxygen therapy (9). Further, COVID-19 patients with severe pneumonia can deteriorate and develop life-threatening acute respiratory failure and ARDS, requiring intensive medical care.

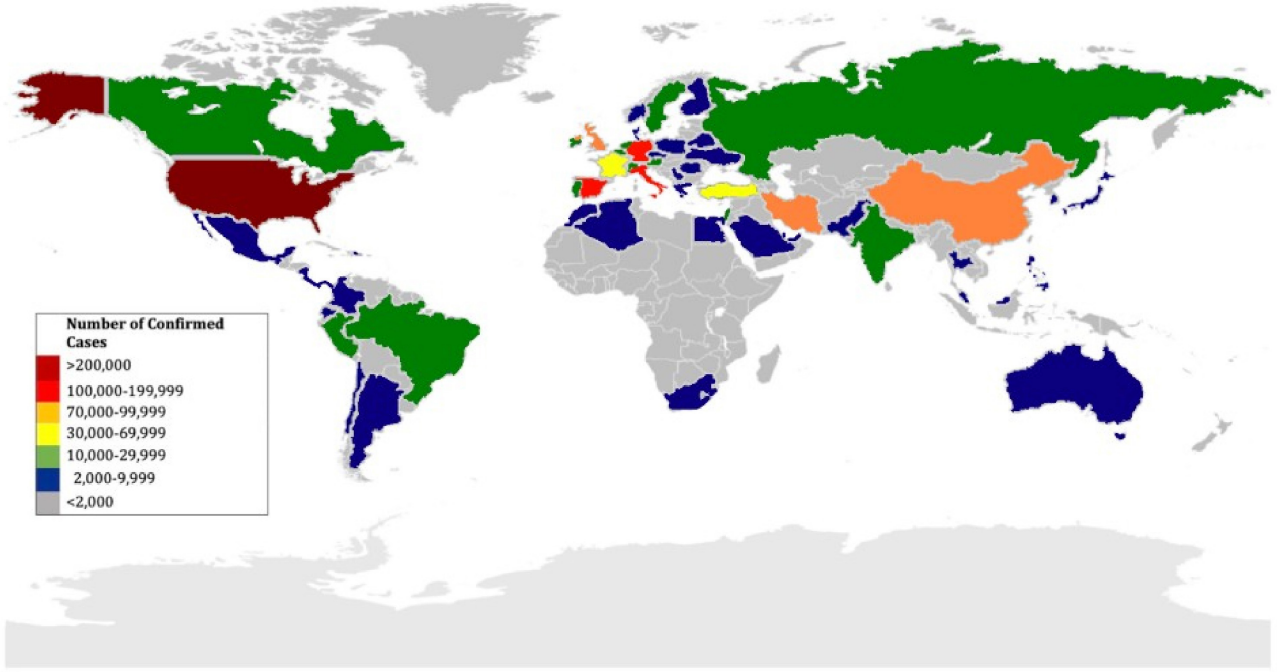


FIGURE 1 | Geographic distribution of countries affected by COVID-19 as of April 16, 2020.

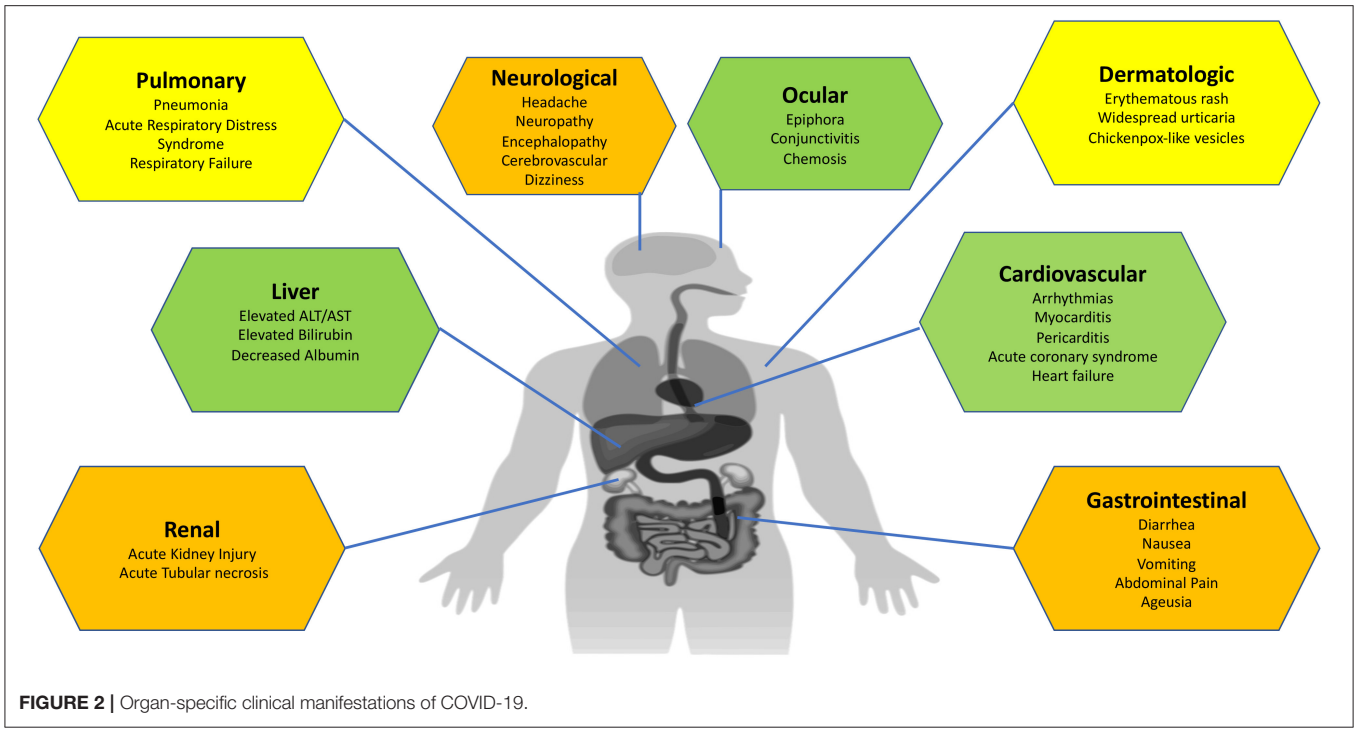


FIGURE 2 | Organ-specific clinical manifestations of COVID-19.

TABLE 1 | Published meta-analyses of Pulmonary Manifestations of COVID-19.

Authors	Number of studies	Number of cases	Fever	Cough	Dyspnea	Sputum production	Sore throat
Long-quan Li et al.	10	1995	88.50%	68.60%	21.90%	28.20%	NR
Rodriguez-Morales et al.	19	656	88.70%	57.60%	45.60%	NR	11.00%
Yang et al.	8	46248	91.0%	67%	30.00%	NR	NR
Cao et al.	31	46,959	87.30%	58.10%	38.30%	NR	12.00%
Borges et al.	61	59,254	82.00%	61.00%	26%	NR	10.00%
Sun et al.	10	50,466	89.00%	72.20%	NR	NR	NR
Fu et al.	43	1,600	83.30%	60.30%	24.90%	26.90%	12.30%
Di Mascio et al.	19	79	82.60%	57.10%	27.00%	NR	NR

A metanalysis ($n = 656$) of observational studies and case reports showed that nearly one third (32.8%) of patients with COVID-19 developed ARDS during their hospital admission (7). Similarly, in a retrospective analysis of clinical findings in 85 patients with confirmed COVID-19, 74.1% of patients developed ARDS during their hospitalization (21). Lai et al. ($n = 72$) identified that about 20% developed ARDS and >25% of patients with COVID-19 required intensive care unit (ICU) admission (22). In one large retrospective study ($n = 710$) by Yang et al., 61.5% of patients with COVID-19 pneumonia died in 28 days with a mean interval from ICU admission to death being 7 days (23).

Comparison of Pulmonary Manifestations Among Covid-19, SARS, and MERS

Given that SARS-CoV-1, SARS CoV-2, and MERS-CoV are all members of the coronavirus family, several comparisons have been made regarding the pulmonary manifestations of these diseases (**Table 2**). The respiratory manifestations of SARS and MERS are very similar to COVID-19. The most common presenting symptoms in patients with COVID-19, SARS, and MERS are cough and dyspnea. Further, in patients with COVID-19, dry cough is present in the early stage of infection, progressing to an expectorant cough with the growing severity of the illness (24). Similarly, in patients with SARS, initial symptoms included cough (61.8%) and dyspnea (40.8%) (24). There are fewer upper airway symptoms that occur in COVID-19 as compared to SARS (25), while patients with MERS may present with hemoptysis, cough, and shortness of breath.

Putative Mechanisms of Pulmonary Injury

It has been well-established that the target of entry for SARS-CoV-2 is the angiotensin-converting enzyme-2 (ACE-2) receptors (**Figure 3**) (3, 26, 27). ACE-2 receptors are expressed in type I & II alveolar cells, and airway epithelial cells (25). The

virus enters these cells using cell-mediated endocytosis and starts a cascade of pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF) (28). When SARS-CoV-2 binds the ACE-2 receptor, it reduces the expression of ACE-2 (29). Interestingly, SARS-CoV-1 and SARS-CoV-2 bind the same ACE-2 receptor; however, SARS-CoV-2 binds this receptor with 10–20 times greater affinity than SARS-CoV-1 (26).

GASTROINTESTINAL MANIFESTATIONS OF COVID-19

Though respiratory symptoms predominate, gastrointestinal (GI) complications from SARS-CoV-2 infection have also been described, and may even precede respiratory symptoms (10, 30). The most frequently reported GI manifestation include nausea, vomiting, diarrhea, and abdominal pain (10, 31–36).

TABLE 2 | Comparison of coronavirus outbreaks between SARS, MERS, and COVID-19.

	SARS	MERS	COVID-19
Year outbreak	2003	2012	2019
Source of outbreak	Bats	Camels, camel products	Bats, seafood
Outbreak location	Guangdong, China	Saudi Arabia	Wuhan, China
Route of transmission	Droplets, contact	Contact	Droplets, contact
Case fatality rate	9.5%	35%	Not yet determined
Basic reproduction number, R0	4.0	1.0	2.0–3.5

In a retrospective study (n = 138) of hospitalized COVID-19 patients, 10% of patients reported both nausea and diarrhea. Abdominal pain and vomiting were recorded in 5 and 3% of patients, respectively (10). Of note, 10% of patients experienced nausea and diarrhea between 1 and 2 days before experiencing respiratory symptoms, suggesting that GI symptoms may atypically present as one of the initial clinical manifestations of COVID-19 (10). Similarly, in a large metanalysis of 10 studies with a total of 1995 cases, diarrhea occurred in 4.8% of cases, while nausea and vomiting occurred in 3.9% of cases (10). Jin et al. also recorded either diarrhea, nausea, or vomiting in 74 of the 651 infected patients reviewed in their study (32). Interestingly, patients who experienced GI symptoms were more likely than those without GI symptoms to have a more severe disease course, characterized by greater degrees of liver insult (17.57 vs. 8.84%), development of ARDS (6.76 vs. 2.08%), and ICU admission requiring mechanical ventilation (6.76 vs. 2.08%) (32). Further, nearly a quarter (22.97%) of the study population who experienced critical illness reported GI symptoms at initial presentation (32).

Possible Mechanisms of GI Manifestations

The fecal-oral route has been proposed as a potential mechanism of GI infection with SARS-CoV-2 (37–39) due to the identification of SARS-CoV-2 RNA in the stool specimens of infected patients (40). Xiao et al. studied the RNA in feces from 73 patients with COVID-19, and 53% of the patients tested positive for SARS-CoV-2 RNA in the stool (41). Additionally, studies have found overexpression of ACE-2 in the epithelial cells of the GI tract, suggesting SARS-CoV-2 replication in the GI tract (42). A case of positive fecal specimen in a symptomatic COVID-19 patient with a negative pharyngeal and sputum specimen has also been published in the literature (43).

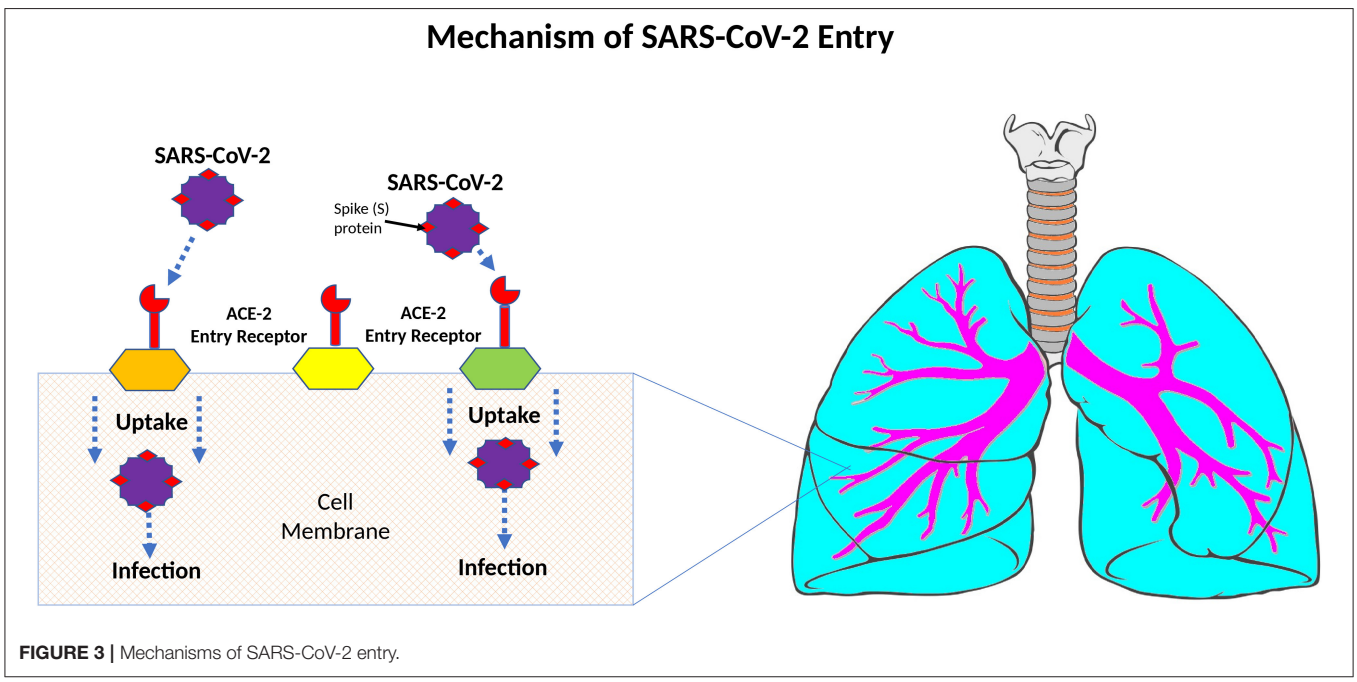


FIGURE 3 | Mechanisms of SARS-CoV-2 entry.

HEPATIC MANIFESTATIONS OF COVID-19

The liver is another organ which can be affected by SARS-CoV-2 (6, 44–46). Commonly reported hepatic manifestations of COVID-19 include elevations in serum levels of alanine transaminase (ALT), aspartate transaminase (AST), and bilirubin, while levels of albumin are decreased (6, 44). In a single-center retrospective study ($n = 99$) in patients with reverse transcription-polymerase chain reaction (RT-PCR) confirmed COVID-19, nearly half (43%) of patients demonstrated abnormal liver chemistries (6). Decreased albumin was noted in 98% of patients, while serum levels of AST, ALT, and bilirubin were elevated in 35, 28, and 18% of patients, respectively. Similarly, in an analysis of 1,099 patients, increased levels of AST were observed in 18.2% of patients with non-severe disease and 39.4% of patients with severe disease, while increased ALT levels were observed in 19.8% of patients with non-severe disease and 28.1% of patients with severe disease (9). The authors in this study used the 2007 American Thoracic Society criteria for community-acquired pneumonia to define COVID-19 disease severity (47). With these results, it appears that the degree of liver injury may be associated with COVID-19 disease severity. In a recent meta-analysis Lippi et al., demonstrated that hepatic factors that were predictive of patients with an unfavorable course of COVID-19 requiring ICU admission included an increase in levels of ALT (1.5–1.8-fold), AST (1.8-fold), total bilirubin (1.2–1.3-fold) and decreased albumin (0.8-fold) (48).

Other studies have demonstrated isolated elevations in AST alone. In a study ($n = 81$) by Shi et al., more than 50% of COVID-19 patients were observed to have elevated levels of AST with normal ALT (44). Similarly, in another study ($n = 41$), 63% of ICU admitted COVID-19 patients had elevated AST vs. only 25% of patients who did not require ICU care (5).

Patients infected with the 2003 SARS-CoV-1 also experienced liver impairment (49–52). Similar to the hepatic injuries associated with COVID-19, the most frequent GI clinical manifestations of SARS included elevations in levels of serum bilirubin, ALT and/or AST, and decreased levels of serum albumin (49–52).

Mechanisms of Hepatic Infection/Injury

The number of studies to better understand the mechanisms of hepatic injury in patients infected with coronaviruses is limited. One proposed mechanism for hepatic injury with SARS-CoV-1 includes heightened inflammatory response to the virus infection (53). This mechanism has been supported by the abnormally high serum levels of cytokines (serum IL-1, IL-6, IL-10) observed in SARS patients and deranged liver chemistries (53). Another proposed mechanism is direct hepatic injury by SARS-CoV-1 via the entry of the ACE-2 receptor on hepatic endothelial cells. Further, SARS-CoV-1 viral particles have also been identified in the liver autopsies of deceased SARS patients and SARS viral genomes have been found by RT-PCR in hepatocytes (54). Contrarily, the proposed mechanism of hepatic injury with MERS-CoV involves dipeptidyl peptidase-4 (DPP-4) as its entry receptor to establish infection in the hepatocytes (55). Several animal and human studies have demonstrated higher DPP-4 expression in liver, and MERS-CoV can infect the liver cells via DPP-4 on the cell surface, causing cell damage and mild to moderate liver injury (56).

Data regarding the mechanism of hepatic injury by SARS-CoV-2 is scarce. It has been proposed that SARS-CoV-2 attaches to ACE-2 as its entry receptor, similar to SARS-CoV-1. A preliminary study by Chai et al. showed over expression of ACE-2 specifically in cholangiocytes, indicating that the virus may potentially bind to cholangiocytes to cause hepatic dysfunction (57). Further, liver biopsy of a deceased COVID-19 patient with deranged liver chemistries showed moderate microvascular steatosis, and mild lobular and portal activity, which was thought to be caused by SARS-CoV-2 infection. However, more studies are needed to further evaluate the mechanism of injury. It is important to mention that concurrent use of hepatotoxic medications in patients with COVID-19 may contribute to liver injury in patients with COVID-19 who are receiving treatment, complicating the discovery of the exact etiology of liver injury (58).

CARDIOVASCULAR MANIFESTATION OF COVID-19

The cardiac manifestations of COVID-19 include cardiac arrhythmias, myocarditis, pericarditis, acute coronary syndrome (ACS), heart failure, cardiogenic shock, and cardiac arrest (**Figure 4**). Though there appears to be no difference in the prevalence of cardiovascular disease (CVD) amongst those with COVID-19 compared to the general population, patients with pre-existing CVD are at higher risk of developing severe COVID-19 (59, 60). A meta-analysis of COVID-19 patients revealed that the prevalence of hypertension, cardio-cerebrovascular disease, and diabetes mellitus was 17.1, 16.4, and 9.7%, respectively. Moreover, the prevalence of hypertension, cardio-cerebrovascular disease, and diabetes mellitus was 2-, 3-, and 2-folds in severe/ICU cases as compared to non-ICU cases, respectively. Additionally, the meta-analysis acknowledged and analyzed viral damage to the heart noting that at least 8% of patients with COVID-19 suffered acute cardiac injury (59). The meta-analysis also evaluated the incidence of myocardial injury in severe/ICU cases and non-ICU cases. Acute cardiac injury was assessed using cardiac markers troponin I/T or CK if troponin I/T were not provided (59). The analysis showed a 13-fold higher incidence of myocardial injury as measured by elevations in Troponin I/T or CK in severe/ICU cases compared to non-ICU COVID-19 cases (59). Additionally, in a separate study ($n = 187$), Guo et al. showed that mortality in COVID-19 patients during hospitalization was greatly associated with presence of CVD and myocardial injury. The study revealed inpatient mortality of 7.62% for patients without underlying CVD and normal troponin T (TnT) levels, 13.33% for those with underlying CVD and normal TnT levels, 37.50% for those without underlying CVD but elevated TnT levels, and 69.44% for those with underlying CVD and elevated TnTs (61). This data indicate that those with cardiovascular comorbidities are

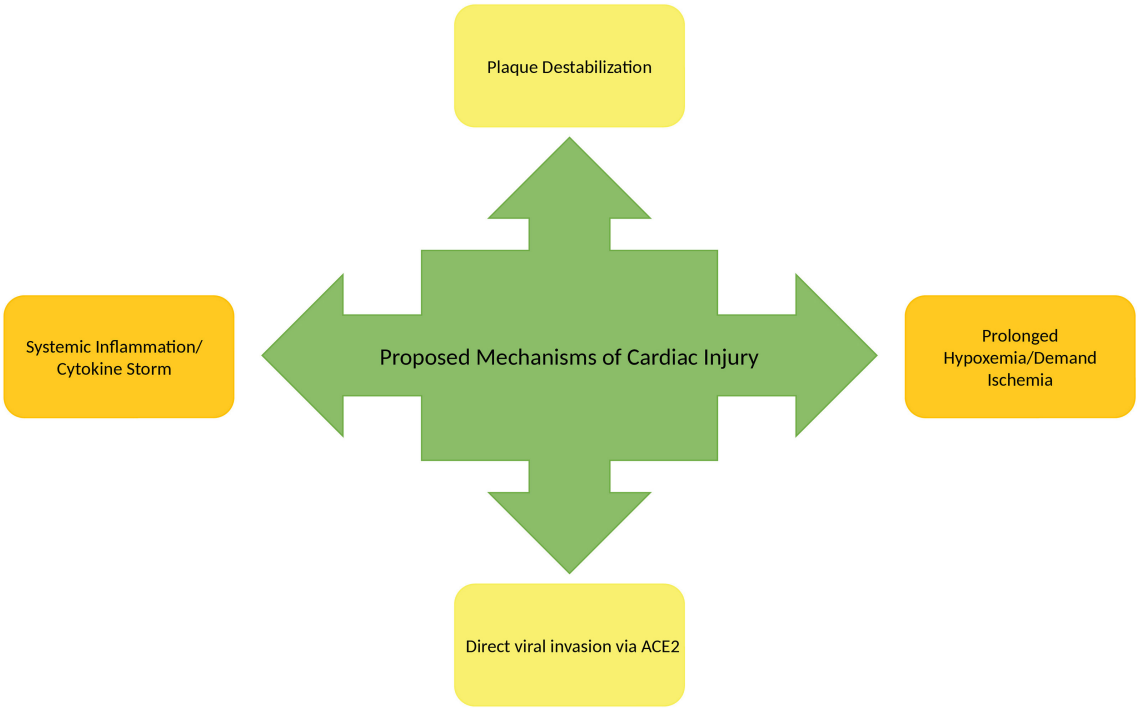


FIGURE 4 | Mechanisms of cardiac injury in COVID-19.

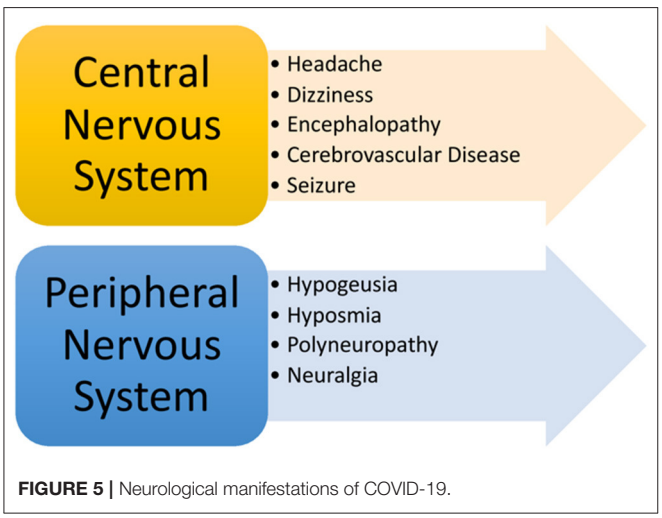


FIGURE 5 | Neurological manifestations of COVID-19.

more likely to have a poor outcome with a more severe COVID-19 course. Similarly, previous SARS outbreaks had increased mortality associated with CVD and diabetes in SARS (59).

In addition to the myocardial injury evidenced by elevations in troponins, another cardiac manifestation of COVID-19 includes arrythmias. In a retrospective study (n = 137) by Lui et al., heart palpitations were reported as an initial symptom in 7.3% of patients with COVID-19 (19). Similarly, 17% of hospitalized COVID-19 patients had unspecified arrhythmias in a separate case series(n = 138) (10). Another study (n = 187) reported ventricular tachycardia/ventricular fibrillation at a rate of 5.9% in hospitalized COVID-19 patients in Wuhan, China (61). Though there has been no biopsy or cardiac magnetic resonance imaging (cMRI) proven fulminant myocarditis or pericarditis, several case series and case reports recognize these as one of the manifestations of COVID-19 based on clinical suspicion and objective data (60, 62). Additionally, development of heart failure or cardiogenic shock was observed in several studies. In a retrospective cohort study of 191 hospitalized COVID-19 patients at two Chinese hospitals, 23% of patients had evidence of heart failure or cardiogenic shock (63).

Mechanisms of Development of Cardiac Manifestations

Though the exact mechanism of myocardial injury, development of heart failure, and cardiogenic shock is unknown, there are a number of proposed mechanisms to consider. One of those mechanisms involves direct cardiac myocyte toxicity associated with viral invasion. Similar to SARS-CoV-1, SARS-CoV-2 has binding affinity for the ACE-2 receptor in myocardial cells. Zou et al. performed mapping of cells in various organ systems expressing ACE-2 with the use of single-cell RNA sequencing. Cells expressing similar or more ACE-2 than lung type II alveolar cells (AT2) were deemed as having the potential for increased vulnerability to SARS-CoV-2 (64). In their study, >7.5% of myocardial cells displayed ACE-2 expression suggesting that the heart may be at high risk for direct cellular toxicity by SARS-CoV-2 entry and replication. This ability to infiltrate cardiac tissues appears to be similar to MERS-CoV and SARS-CoV-1. In

an animal model study using transgenic mice, MERS-CoV RNA was detectable in the heart (65). Similarly, in a study from the Toronto SARS outbreak, RNA of SARS-CoV-1 was found in 35% of cardiac tissues on autopsy (66).

Another proposed mechanism of cardiac effects of COVID-19 is heightened release of pro-inflammatory cytokines through activation of the innate and adaptive immune system. The increased production of cytokines such as IL-6, IL-10, and TNF-α can lead to multiorgan failure. In the past, IL-6 has been associated with cardiomyopathy. Additionally, this inflammatory state can promote and contribute to atherosclerotic plaque rupture (63) and acute coronary syndrome. In the setting of critical illness due to COVID-19, prolonged exposure to catecholamines and cytokine storm as a response to infection can result in myocardial damage as well as stress-induced cardiomyopathy (59).

Due to the respiratory sequelae of COVID-19, some patients suffer from hypoxemia which is another proposed mechanism of cardiac injury. As prolonged hypoxemia results in reduced cellular capacity to metabolize aerobically, cells are subsequently switched to anaerobic metabolism. Anaerobic metabolism produces a more acidotic state intracellularly due to increased lactic acid production. Subsequently, increased free radical production and direct destruction of phospholipid cell membranes occur (59). Hypoxemia can also increase calcium ion influx which may lead to cardiac myocyte apoptosis (59). Demand ischemia associated with critical illness can produce similar mechanisms of cardiac injury.

Though more research is needed to further assess the pathogenesis associated with COVID-19 myocardial injury, data obtained thus far indicates the presence of viral-related heart damage. This damage manifests in a variety of ways including evidence of arrythmias, pericarditis, myocarditis, heart failure, cardiac shock, and cardiac arrest (59, 61). Finally, current data suggests cardiovascular disease, cardiac manifestations, and cardiac injury in the setting of COVID-19 are clinically relevant predictors of overall disease severity and mortality (59, 61).

RENAL MANIFESTATIONS OF COVID-19

Another organ system affected by SARS-CoV-2 is the renal system with development of acute kidney injury (AKI). This can occur in those with chronic kidney disease (CKD) as well as those with no evidence of prior renal impairment. Though acknowledged as a rare occurrence in SARS, it appears that AKI may be more common in COVID-19 (67, 68). In a single center case series ($n = 138$) assessing clinical characteristics of patients with COVID-19, Wang et al. noted that ∼4% of these patients with COVID-19 had an AKI (10). Huang et al. determined in a separate study of 41 COVID-19 positive patients that ∼7% had evidence of an AKI (69). In a small Washington state study consisting of 21 critically ill COVID-19 patients, 19.1% ($n = 6$) had acute kidney failure according to KDIGO guidelines (70). Analysis of 51 critically ill COVID-19 patients in a Wuhan, China study showed 29% ($n = 15$) developed an AKI (23). Similarly, during the SARS outbreak, AKI was also observed. In a study performed by Chu et al., 6.7% of 537 patients with SARS developed an AKI in the setting of normal Cr on admission (67). Additionally, Chu et al. revealed a significantly higher mortality rate (91.7%) associated with those having evidence of renal impairment compared to those with normal renal function in the setting of SARS (8.8%). In Wang's study of COVID-19 patients, AKI was observed more in ICU than non-ICU patients. This might indicate that severity of illness progression associated with COVID-19 may be significantly impacted by the presence of renal impairment.

Mechanism of Injury

Several potential pathophysiological explanations have been suggested to explain renal impairment in COVID-19. ACE-2 receptor have been shown to be highly expressed in the proximal tubules and urothelial cells of the bladder on single cell RNA sequencing (63). The increased susceptibility of the kidney to viral entry associated with ACE-2 expression make it a possible target for direct cellular toxicity. Moreover, SARS-CoV-2 has a significantly higher affinity for the ACE-2 receptor which could explain the higher incidence of AKI in COVID-19. Another possible mechanism of AKI in COVID-19 is significantly higher immune response to infection and multiorgan failure. SARS-CoV-2 induces the release of inflammatory cytokines IL-2, IL-7, IL-10 which are believed to be involved in the pathology of AKI (71). Additionally, in critically ill patients, the presence of hypovolemia, rhabdomyolysis, hypoxemia, sepsis, and septic shock associated with this viral illness are likely to contribute significantly to renal impairment. Furthermore, the possibility that the etiology of AKIs seen in COVID-19 patients is multifactorial should also be considered.

NEUROLOGICAL

More recently, a wide range of neurological complications have been reported in patients with COVID-19 suggesting that SARS-CoV-2 may affect both the central and peripheral nervous system (**Figure 5**). Commonly reported central nervous system (CNS) manifestations include headache, acute cerebrovascular disease, dizziness, and encephalopathy. In a retrospective study ($n = 214$) of confirmed COVID-19 patients, 36.4% of subjects collectively experienced either dizziness, headache, cerebrovascular disease, and/or reduced consciousness (72). Headache appears to be one of the most common CNS symptoms, which has been reported at a rate of 6–13% in patients with COVID-19 (5, 6, 23, 73). Dizziness occurred in nearly 9–17% of patients based on recent studies (10, 72), while reduced levels of consciousness and confusion occurred in 7.5 and 9% of COVID-19 patients, respectively (6). In a recent case series ($n = 58$) of COVID-19 patients who developed ARDS, several neurologic findings, including encephalopathy, confusion, agitation and corticospinal tract signs were reported in 84% of cases. However, it is unclear whether or not these neurological signs and symptoms were directly related to infection with SARS-CoV-2, medication withdrawal, or cytokine effects (74). To a lesser extent, seizure has also been reported in a minority of cases

(0.5%) (72). Similarly, rare cases of confirmed viral encephalitis and meningitis have been described in small case reports of patients with SARS-CoV-2 detected in the cerebrospinal fluid (75, 76).

Cerebrovascular disease represents another cluster of CNS manifestations of COVID-19 that have been cited in the literature. In a retrospective, observational study (n = 221) of patients with COVID-19 in Wuhan China, acute ischemic stroke (confirmed on head CT) was reported in 5% of subjects. Additionally, cerebral venous sinus thrombosis (confirmed with CT venography), and cerebral hemorrhage both occurred at a rate of 0.5% in this cohort (77). Another study demonstrated that ischemic stroke and cerebral hemorrhage (both confirmed on head CT) were collectively noted in 2.8% of 214 patients with COVID-19 (72). Even further, one case report described a case of acute necrotizing hemorrhagic encephalopathy associated with COVID-19 in a middle aged female who presented with acute encephalopathy (78).

Based on recent studies, neurologic manifestations appear to occur more frequently in patients with severe disease courses (79). Mao et al. demonstrated that neurological manifestations (specifically cerebrovascular disease, reduced consciousness and myopathy) occurred more frequently (45.5 vs. 30%) in patients with severe disease compared to those with non-severe disease. Of note, those with more severe disease (as defined by the previously described American Thoracic Society guidelines) were also noted to have a greater burden of co-morbidities. However, it is unclear whether these neurologic manifestations hold any prognostic value in regards to COVID-19 mortality (72).

In addition to CNS manifestations, peripheral nervous system (PNS) findings related to COVID-19 have been described in the literature. Clinical data has demonstrated that patients with COVID-19 may experience changes in smell and/or taste, in addition to polyneuropathy and/or even neuralgia. In a retrospective study of COVID-19 patients, hypogeusia, and hyposmia occurred in 5.6 and 5.1% of patients, respectively, while neuralgia or peripheral nerve pain occurred in 2.3% of the study patients. In the same study, changes in smell occurred in 12% of patients prior to any respiratory symptoms (80), suggesting that hyposmia may uncommonly precede development of any respiratory symptoms. Other studies have demonstrated similar findings (72, 81). Another study (n = 60) demonstrated that reduced smell function was prominent (98%) among patients diagnosed with COVID-19, but concluded that changes in smell did not hold any prognostic value (82). In addition to changes in smell, one study showed that up to 88% of patients with COVID-19 experienced changes (diminished or complete loss) in taste before and during their disease course (83). As such, it is reasonable to consider change in smell and/or taste as potential warning signs for COVID-19, while neuralgia and peripheral neuropathy may be considered disease manifestations that may along the course of infection. Clinicians should conduct a thorough neurological history and physical to identify early signs and symptoms of patients who many warrant COVID-19 testing (83).

OCULAR/OPHTHALMIC

Healthcare professionals can get exposed to ocular secretion of the infected patients and these secretions could become a fomite for viral spread. Ocular manifestations such as conjunctivitis, retinitis, anterior uveitis, and optic neuritis have been reported due to infections from the coronaviruses in the past (84–86). However, there is paucity of literature regarding the ocular manifestations of COVID-19, possibly because these manifestations are under-recognized and under-reported.

In a case series of 36 patients with confirmed COVID-19, nearly one third (31.6%) of patients developed ocular manifestations such as chemosis, epiphora, and conjunctival congestion. Interestingly patients with ocular manifestations experienced a severe disease course. Loon et al. published a case series of patients with suspected and probable SARS infection who had tear samples collected and analyzed by PCR. Using WHO case definitions of suspected and probable cases, eight patients were classified as probable SARS (based on chest imaging suggestive of pneumonia or ARDS) and 28 were classified as suspects of SARS (anyone experiencing fever $>100.4°$F, respiratory symptoms and known contact with a confirmed case of SARS) (87). Of 36 subjects tested, three with probable SARS had positive SARS-CoV results from their tear samples suggesting that SARS-CoV-1 can exist in tears and may potentially be a source of spread among healthcare workers and inoculating patients (88). Similarly, another earlier predecessor of the SARS-CoV-2, the human CoV-NL63 virus was isolated from nasopharyngeal aspirate from an infant who had conjunctivitis and bronchiolitis (89). Another retrospective study of 18 children with acute respiratory tract infection due to CoV-NL63 showed that three patients also developed conjunctivitis (90). However, some controversy endures as some authors have proclaimed that ACE-2 receptors predominantly exist in the posterior eye, which would not account for the cases of anterior uveitis and conjunctivitis related to SARS-CoV-1 (91).

CUTANEOUS MANIFESTATIONS OF COVID-19

Cutaneous findings have also been reported as a manifestation of COVID-19. While there is little data regarding the topic, currently reported manifestations include erythematous rash, vesicular lesions, and urticaria. In a small analysis (n = 88) of patients who tested positive for COVID-19, nearly 20% of patients developed skin findings (92). Of the 88 positive patients, eight presented with skin findings at disease onset, while 10 developed skin findings during hospitalization. Nearly 16% developed an erythematous rash, while 1.1 and 3.4%

developed vesicular lesions and urticaria, respectively. The most commonly affected cutaneous region was the trunk and most lesions resolved within a few days (92). While preliminary data exists, many more studies are needed to provide additional information regarding the dermatologic manifestations of COVID-19.

Asymptomatic Covid-19 Patients

While SARS-CoV-2 has been shown to affect various organs with a variety of clinical manifestations, some patients with RT-PCR detected SARS-CoV-2 remain completely asymptomatic. A number of studies report a wide incidence rate of asymptomatic infections, ranging from 1.6 to 56.6% (93–98). According to these studies, asymptomatic patients typically experience none of the aforementioned clinical signs and/or symptomology. Even further, this subgroup of patients have little to no abnormalities on radiological imaging. While some with asymptomatic infection may develop into symptomatic cases, most progress without clinical deterioration. Hu et al. conducted a study ($n = 24$) in asymptomatic patients (no symptoms at the time of screening) who tested positive for COVID-19. Of the 24 patients in the study, mortality was not observed in any of the patients, however 20% later developed fever, cough, and/or fatigue during the course of hospitalization (99).

Similarities and Differences in Comorbid Health Conditions in Covid-19, MERS, and SARS

It has been well-established that patients with pre-existing comorbidities generally experience worse health outcomes (higher rates of mortality, ICU admission, mechanical ventilation) compared to patients who do not have any underlying health conditions. This predisposition to more severe disease can be attributed to the negative impact that comorbidities have on the individual's immune system and subsequent decreased ability to fight infection (100). Prior to the emergence of COVID-19, previous studies have substantiated the notion that patients with SARS and patients with MERS who also had comorbidities generally experienced poorer health outcomes (101). These comorbidities commonly included heart disease, diabetes mellitus (DM), chronic obstructive pulmonary disease (COPD), cancer, chronic renal disease, hypertension, ischemic heart disease, congestive heart failure, asthma, and cerebrovascular accident (CVA). In an analytical study ($n = 115$) of patients with SARS, diabetes, and heart disease were each found to be independent risk factors for mortality. Specifically, patients with heart disease and/or diabetes conferred a 12.5-time higher risk of mortality (102). Another retrospective case series ($n = 144$) showed that SARS patients with a diagnosis of diabetes had a 3-fold increased the risk of death, ICU admission, or mechanical ventilation, while SARS patients with other comorbid conditions like COPD, heart disease or cancer had a 2.5 increased risk. In the same study, only one of the 144 subjects, a patient with no known comorbidities (former smoker), experienced mortality (103).

Similarly, in patients with MERS, pre-existing health conditions were shown to impact the severity of the disease course. In a 2013 Saudi Arabia study ($n = 47$) of patients with MERS, nearly 64% of the study population with diagnosed comorbidities (diabetes, hypertension, cardiac disease, and chronic renal disease) experienced mortality while only 14% of the study population without comorbidities died (104). Another study ($n = 1{,}743$), which investigated the impact of comorbidity on mortality rate in MERS patients, found that patients without any comorbidities had a higher 21-day survival rate compared to patients with known comorbidities. Further, MERS patients with comorbidities had a 4-fold risk for fatal health outcomes compared to those without comorbidities (105).

Similar to patients with MERS and SARS, disparities are seen between health outcomes of COVID-19 patients with pre-existing health conditions and those without (12). In a retrospective analysis ($n = 138$) of patients with COVID-19, nearly half (46.4%) of patients had an underlying health condition. Even further, those patients burdened with multiple comorbidities (72.2%) were more likely to require ICU admission compared to those with no comorbidities (37.3%) (10). While it is well-known that having comorbidities establishes an increased risk of disease severity, few studies have previously identified which conditions confer the greater risk of COVID-19 disease severity. In a large metanalysis ($n = 1{,}558$) of patients with COVID-19, hypertension, DM, COPD, heart disease, and cerebrovascular disease were all found to be independent risk factors for severe disease (defined by either an ICU admission or severity of symptoms), while patients with liver disorders, cancer, or kidney disease experienced no increased risk (106). In another metanalysis ($n = 1{,}813$), Jain et al. showed that patients with COVID-19 who also had underlying COPD, hypertension, and/or cardiovascular disease had a greater risk of requiring ICU admission or experiencing severe disease (107). Even further, COPD was shown to be the greatest predictive comorbid risk factor for severe disease and ICU admission followed by cardiovascular disease and hypertension (107).

CONCLUSION

COVID-19 has become a pandemic and a public health emergency, affecting more individuals than previous coronavirus outbreaks with SARS and MERS. The clinical manifestations of COVID-19 are primarily related to the pulmonary system, and include dyspnea, cough with sputum production, fatigue and in severe cases, ARDS, respiratory failure, and even death. Extrapulmonary clinical manifestations of COVID-19 exist and affect multiple other organs and organ systems including cardiovascular, renal, hepatic, gastrointestinal, ocular, dermatologic, and neurological systems. Clinicians should be aware of the variable organ and organ systems affected in patients with COVID-19 and the potential disease course in patients.

AUTHOR CONTRIBUTIONS

KJ, CHa, JC, CHu, HG, and AP equally contributed to this paper with conception and design of the study, literature review and analysis, drafting and critical revision and editing, and final approval of the final version. All authors contributed to the article and approved the submitted version.

REFERENCES

1. Ksiazek TG, Erdman D, Goldsmith CS, Zaki SR, Peret T, Emery S, et al. A novel coronavirus associated with severe acute respiratory syndrome. *N Engl J Med.* (2003) 348:1953–66. doi: 10.1056/NEJMoa030781
2. Hui DS, Azhar EI, Kim YJ, Memish ZA, Oh MD, Zumla A. Middle East respiratory syndrome coronavirus: risk factors and determinants of primary, household, and nosocomial transmission. *Lancet Infect Dis.* (2018) 18:e217–27. doi: 10.1016/S1473-3099(18)30127-0
3. Velavan TP, Meyer CG. The COVID-19 epidemic. *Trop Med Int Health.* (2020) 25:278–80. doi: 10.1111/tmi.13383
4. Li Q, Guan X, Wu P, Wang X, Zhou L, Tong Y, et al. Early transmission dynamics in Wuhan, China, of novel coronavirus-infected pneumonia. *N Engl J Med.* (2020) 382:1199–207. doi: 10.1056/NEJMoa2001316
5. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet.* (2020) 395:497–506. doi: 10.1016/S0140-6736(20)30183-5
6. Chen N, Zhou M, Dong X, Qu J, Gong F, Han Y, et al. Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study. *Lancet.* (2020) 395:507–13. doi: 10.1016/S0140-6736(20)30211-7
7. Rodriguez-Morales AJ, Cardona-Ospina JA, Gutierrez-Ocampo E, Villamizar-Pena R, Holguin-Rivera Y, Escalera-Antezana JP, et al. Clinical, laboratory and imaging features of COVID-19: A systematic review and meta-analysis. *Travel Med Infect Dis.* (2020) 34:101623. doi: 10.1016/j.tmaid.2020.101623
8. Worldometers.info. Dover, DE (2020) Available online at: https://www.worldometers.info/coronavirus/#countries (accessed July 18, 2020).
9. Guan WJ, Ni ZY, Hu Y, Liang WH, Ou CQ, He JX, et al. Clinical characteristics of coronavirus disease 2019 in China. *N Engl J Med.* (2020) 382:1708–1720. doi: 10.1056/NEJMoa2002032
10. Wang D, Hu B, Hu C, Zhu F, Liu X, Zhang J, et al. Clinical characteristics of 138 hospitalized patients with 2019. novel coronavirus-infected pneumonia in Wuhan, China. *JAMA.* (2020) 323:1061–9. doi: 10.1001/jama.2020.1585
11. Zhang JJ, Dong X, Cao YY, Yuan YD, Yang YB, Yan YQ, et al. Clinical characteristics of 140 patients infected with SARS-CoV-2 in Wuhan, China. *Allergy.* (2020) 75:1730–41. doi: 10.1111/all.14238
12. Yang J, Zheng Y, Gou X, Pu K, Chen Z, Guo Q, et al. Prevalence of comorbidities in the novel Wuhan coronavirus (COVID-19) infection: a systematic review and meta-analysis. *Int J Infect Dis.* (2020) 94:91–5. doi: 10.1016/j.ijid.2020.03.017
13. Cao Y, Liu X, Xiong L, Cai K. Imaging and clinical features of patients with 2019 novel coronavirus SARS-CoV-2: a systematic review and meta-analysis. *J Med Virol.* (2020). doi: 10.1002/jmv.25822. [Epub ahead of print].
14. Borges do Nascimento IJ, Cacic N, Abdulazeem HM, von Groote TC, Jayarajah U, Weerasekara I, et al. Novel coronavirus infection (COVID-19) in humans: a scoping review and meta-analysis. *J Clin Med.* (2020) 9:941. doi: 10.2139/ssrn.3550028
15. Sun P, Qie S, Liu Z, Ren J, Li K, Xi J. Clinical characteristics of hospitalized patients with SARS-CoV-2 infection: A single arm meta-analysis. *J Med Virol.* (2020) 92:612–7. doi: 10.1002/jmv.25735
16. Fu L, Wang B, Yuan T, Chen X, Ao Y, Fitzpatrick T, et al. Clinical characteristics of coronavirus disease 2019. (COVID-19) in China: a systematic review and meta-analysis. *J Infect.* (2020) 80:656–65. doi: 10.1016/j.jinf.2020.03.041
17. Di Mascio D, Khalil A, Saccone G, Rizzo G, Buca D, Liberati M, et al. Outcome of coronavirus spectrum infections (SARS, MERS, COVID 1−19) during pregnancy: a systematic review and meta-analysis. *Am J Obstet Gynecol MFM.* (2020) 2:100107. doi: 10.1016/j.ajogmf.2020.100107
18. Tian S, Hu N, Lou J, Chen K, Kang X, Xiang Z, et al. Characteristics of COVID-19 infection in Beijing. *J Infect.* (2020) 80:401–6. doi: 10.1016/j.jinf.2020.02.018
19. Liu K, Fang YY, Deng Y, Liu W, Wang MF, Ma JP, et al. Clinical characteristics of novel coronavirus cases in tertiary hospitals in Hubei Province. *Chin Med J.* (2020) 133:1025–31. doi: 10.1097/CM9.0000000000000744
20. Meo SA, Alhowikan AM, Al-Khlaiwi T, Meo IM, Halepoto DM, Iqbal M, et al. Novel coronavirus 2019-nCoV: prevalence, biological and clinical characteristics comparison with SARS-CoV and MERS-CoV. *Eur Rev Med Pharmacol Sci.* (2020) 24:2012–9. doi: 10.26355/eurrev_202002_20379
21. Du Y, Tu L, Zhu P, Mu M, Wang R, Yang P, et al. Clinical features of 85 fatal cases of COVID-19 from Wuhan: a retrospective observational study. *Am J Respir Crit Care Med.* (2020) 201:1372–9. doi: 10.2139/ssrn.3546088
22. Lai CC, Shih TP, Ko WC, Tang HJ, Hsueh PR. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and coronavirus disease-2019 (COVID-19): the epidemic and the challenges. *Int J Antimicrob Agents.* (2020) 55:105924. doi: 10.1016/j.ijantimicag.2020.105924
23. Yang X, Yu Y, Xu J, Shu H, Xia J, Liu H, et al. Clinical course and outcomes of critically ill patients with SARS-CoV-2 pneumonia in Wuhan, China: a single-centered, retrospective, observational study. *Lancet Respir Med.* (2020) 8:475–81. doi: 10.1016/S2213-2600(20)30079-5
24. Xu J, Zhao S, Teng T, Abdalla AE, Zhu W, Xie L, et al. Systematic comparison of two animal-to-human transmitted human coronaviruses: SARS-CoV-2 and SARS-CoV. *Viruses.* (2020) 12:244. doi: 10.3390/v12020244
25. Li YC, Bai WZ, Hashikawa T. The neuroinvasive potential of SARS-CoV2 may play a role in the respiratory failure of COVID-19 patients. *J Med Virol.* (2020) 92:552–55. doi: 10.1002/jmv.25728
26. Wrapp D, Wang N, Corbett KS, Goldsmith JA, Hsieh CL, Abiona O, et al. Cryo-EM structure of the 2019-nCoV spike in the prefusion conformation. *Science.* (2020) 367:1260–3. doi: 10.1126/science.abb2507
27. Sun P, Lu X, Xu C, Sun W, Pan B. Understanding of COVID-19 based on current evidence. *J Med Virol.* (2020) 92:548–51. doi: 10.1002/jmv.25722
28. Conti P, Ronconi G, Caraffa A, Gallenga CE, Ross R, Frydas I, et al. Induction of pro-inflammatory cytokines (IL-1 and IL-6) and lung inflammation by Coronavirus-19 (COVI-19 or SARS-CoV-2): anti-inflammatory strategies. *J Biol Regul Homeost Agents.* (2020) 34:1. doi: 10.23812/CONTI-E
29. Yang JM, Meng X, Xue F, Zhang Y, Zhang C. [ACE2 in the context of 2019-nCoV infection: friend or foe?]. *Zhonghua Xin Xue Guan Bing Za Zhi.* (2020) 48:E012. doi: 10.3760/cma.j.cn112148-20200303-00149
30. Kopel J, Perisetti A, Gajendran M, Boregowda U, Goyal H. Clinical insights into the gastrointestinal manifestations of COVID-19. *Digestive Dis Sci.* (2020) 65:1932–9. doi: 10.1007/s10620-020-06362-8
31. Zhou Z, Zhao N, Shu Y, Han S, Chen B, Shu X. Effect of gastrointestinal symptoms on patients infected with COVID-19. *Gastroenterology.* (2020) 158:2294–7. doi: 10.1053/j.gastro.2020.03.020
32. Jin X, Lian JS, Hu JH, Gao J, Zheng L, Zhang YM, et al. Epidemiological, clinical and virological characteristics of 74 cases of coronavirus-infected disease 2019. (COVID-19) with gastrointestinal symptoms. *Gut.* (2020) 69:1002–9. doi: 10.1136/gutjnl-2020-320926
33. Perisetti A, Gajendran M, Boregowda U, Bansal P, Goyal H. COVID-19 and gastrointestinal endoscopies: current insights and emergent strategies. *Dign Endosc.* (2020). doi: 10.1111/den.13693. [Epub ahead of print].
34. Aloysius MM, Thatti A, Gupta A, Sharma N, Bansal P, Goyal H. COVID-19 presenting as acute pancreatitis. *Pancreatology.* (2020) 20:1026–7. doi: 10.1016/j.pan.2020.05.003
35. Gadiparthi C, Perisetti A, Sayana H, Tharian B, Inamdar S, Korman A. Gastrointestinal bleeding in patients with severe SARS-CoV-2. *Am J Gastroenterol.* (2020). doi: 10.14309/ajg.0000000000000719. [Epub ahead of print].

36. Aziz M, Perisetti A, Lee-Smith WM, Gajendran M, Bansal P, Goyal H. Taste changes (Dysgeusia) in COVID-19: a systematic review and metaanalysis. *Gastroenterology*. (2020). doi: 10.1053/j.gastro.2020.05.003
37. Yeo C, Kaushal S, Yeo D. Enteric involvement of coronaviruses: is faecal-oral transmission of SARS-CoV-2 possible? *Lancet Gastroenterol Hepatol.* (2020) 5:335–7. doi: 10.1016/S2468-1253(20)30048-0
38. Perisetti A, Gajendran M, Goyal H. Putative mechanisms of diarrhea in COVID-19. *Clin Gastroenterol Hepatol.* (2020). doi: 10.1016/j.cgh.2020.06.008. [Epub ahead of print].
39. Ramachandran P, Onukogu I, Ghanta S, Gajendran M, Perisetti A, Goyal H, et al. Gastrointestinal symptoms and outcomes in hospitalized COVID-19 patients. *Dign Dis.* (2020). doi: 10.1159/000509774. [Epub ahead of print].
40. Wong SH, Lui RN, Sung JJ. Covid-19 and the digestive system. *J Gastroenterol Hepatol.* (2020) 35:744–8. doi: 10.1111/jgh.15047
41. Xiao F, Tang M, Zheng X, Liu Y, Li X, Shan H. Evidence for gastrointestinal infection of SARS-CoV-2. *Gastroenterology.* (2020) 158:1831–3.e3. doi: 10.1101/2020.02.17.20023721
42. Wan Y, Shang J, Graham R, Baric RS, Li F. Receptor recognition by the novel coronavirus from Wuhan: an analysis based on decade-long structural studies of SARS coronavirus. *J Virol.* (2020) 94, e00127-20. doi: 10.1128/JVI.00127-20
43. Chen L, Lou J, Bai Y, Wang M. COVID-19 disease with positive fecal and negative pharyngeal and sputum viral tests. *Am J Gastroenterol.* (2020) 115:790. doi: 10.14309/ajg.0000000000000610
44. Shi H, Han X, Jiang N, Cao Y, Alwalid O, Gu J, et al. Radiological findings from 81 patients with COVID-19 pneumonia in Wuhan, China: a descriptive study. *Lancet Infect Dis.* (2020) 20, 425–34. doi: 10.1016/S1473-3099(20)30086-4
45. Ramachandran P, Perisetti A, Gajendran M, Chakraborti A, Narh JT, Goyal H. Increased serum aminotransferase activity and clinical outcomes in Coronavirus disease 2019. *J Clin Exp Hepatol.* (2020). doi: 10.1016/j.jceh.2020.06.009. [Epub ahead of print].
46. Boregowda U, Aloysius MM, Perisetti A, Gajendran M, Bansal P, Goyal H. Serum activity of liver enzymes is associated with higher mortality in COVID-19: a systematic review and meta-analysis. *Front Med.* (2020) 7:431. doi: 10.3389/fmed.2020.00431
47. Metlay JP, Waterer GW, Long AC, Anzueto A, Brozek J, Crothers K, et al. Diagnosis and treatment of adults with community-acquired pneumonia. an official clinical practice guideline of the American thoracic society and infectious diseases society of America. *Am J Respir Crit Care Med.* (2019) 200:e45–67. doi: 10.1164/rccm.201908-1581ST
48. Lippi G, Plebani M. Laboratory abnormalities in patients with COVID-2019 infection. *Clin Chem Lab Med.* (2020) 58:1131–4. doi: 10.1515/cclm-2020-0198
49. Wu KL, Lu SN, Changchien CS, Chiu KW, Kuo CH, Chuah SK, et al. Sequential changes of serum aminotransferase levels in patients with severe acute respiratory syndrome. *Am J Trop Med Hyg.* (2004) 71:125–8. doi: 10.4269/ajtmh.2004.71.125
50. Zhao LF, Xing HC, Xu LP. [Effect of SARS-associated coronavirus on peripheral blood picture and liver function]. *Zhongguo Wei Zhong Bing Ji Jiu Yi Xue.* (2004) 16:660–3.
51. Duan ZP, Chen Y, Zhang J, Zhao J, Lang ZW, Meng FK, et al. [Clinical characteristics and mechanism of liver injury in patients with severe acute respiratory syndrome]. *Zhonghua Gan Zang Bing Za Zhi.* (2003) 11:493–6.
52. Guan YJ, Tang XP, Yin CB, Yi ZQ. [Study on the damage of liver in patients with SARS]. *Zhongguo Wei Zhong Bing Ji Jiu Yi Xue.* (2004) 16:267–70.
53. Duan XF, Liu Z, Hao R, Luo L, Zhang YN. [The dynamic change of liver injury in patients with severe acute respiratory syndrome]. *Zhonghua Gan Zang Bing Za Zhi.* (2004) 12:439.
54. Ding Y, He L, Zhang Q, Huang Z, Che X, Hou J, et al. Organ distribution of severe acute respiratory syndrome (SARS) associated coronavirus (SARS-CoV) in SARS patients: implications for pathogenesis and virus transmission pathways. *J Pathol.* (2004) 203:622–30. doi: 10.1002/path.1560
55. Raj VS, Mou H, Smits SL, Dekkers DH, Muller MA, Dijkman R, et al. Dipeptidyl peptidase 4 is a functional receptor for the emerging human coronavirus-EMC. *Nature.* (2013) 495:251–4. doi: 10.1038/nature12005
56. Zhao G, Jiang Y, Qiu H, Gao T, Zeng Y, Guo Y, et al. Multi-organ damage in human dipeptidyl peptidase 4 transgenic mice infected with middle east respiratory syndrome-coronavirus. *PLoS ONE.* (2015) 10:e0145561. doi: 10.1371/journal.pone.0145561
57. Chai X, Hu L, Zhang Y, Han W, Lu Z, Ke A, et al. Specific ace2 expression in cholangiocytes may cause liver damage after 2019-nCoV Infection. *bioRxiv*. (2020). doi: 10.1101/2020.02.03.931766
58. Zhang C, Shi L, Wang FS. Liver injury in COVID-19: management and challenges. *Lancet Gastroenterol Hepatol.* (2020) 5:428–30. doi: 10.1016/S2468-1253(20)30057-1
59. Li B, Yang J, Zhao F, Zhi L, Wang X, Liu L, et al. Prevalence and impact of cardiovascular metabolic diseases on COVID-19 in China. *Clin Res Cardiol.* (2020) 109:531–8. doi: 10.1007/s00392-020-01626-9
60. Ruan Q, Yang K, Wang W, Jiang L, Song J. Clinical predictors of mortality due to COVID-19 based on an analysis of data of 150 patients from Wuhan, China. *Intensive Care Med.* (2020) 46:846–8. doi: 10.1007/s00134-020-06028-z
61. Guo T, Fan Y, Chen M, Wu X, Zhang L, He T, et al. Cardiovascular implications of fatal outcomes of patients with coronavirus disease 2019. (COVID-19). *JAMA Cardiol.* (2020) 5:1–8. doi: 10.1001/jamacardio.2020.1017
62. Hu H, Ma F, Wei X, Fang Y. Coronavirus fulminant myocarditis saved with glucocorticoid and human immunoglobulin. *Eur Heart J.* (2020) ehaa190. doi: 10.1093/eurheartj/ehaa190
63. Zhou F, Yu T, Du R, Fan G, Liu Y, Liu Z, et al. Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: a retrospective cohort study. *Lancet.* (2020) 395:1054–62. doi: 10.1016/S0140-6736(20)30566-3
64. Zou X, Chen K, Zou J, Han P, Hao J, Han Z. Single-cell RNA-seq data analysis on the receptor ACE2 expression reveals the potential risk of different human organs vulnerable to 2019-nCoV infection. *Front Med.* (2020) 14:185–92. doi: 10.1007/s11684-020-0754-0
65. Agrawal AS, Garron T, Tao X, Peng BH, Wakamiya M, Chan TS, et al. Generation of a transgenic mouse model of Middle East respiratory syndrome coronavirus infection and disease. *J Virol.* (2015) 89:3659–70. doi: 10.1128/JVI.03427-14
66. Oudit GY, Kassiri Z, Jiang C, Liu PP, Poutanen SM, Penninger JM, et al. SARS-coronavirus modulation of myocardial ACE2 expression and inflammation in patients with SARS. *Eur J Clin Invest.* (2009) 39:618–25. doi: 10.1111/j.1365-2362.2009.02153.x
67. Chu KH, Tsang WK, Tang CS, Lam MF, Lai FM, To KF, et al. Acute renal impairment in coronavirus-associated severe acute respiratory syndrome. *Kidney Int.* (2005) 67:698–705. doi: 10.1111/j.1523-1755.2005.67130.x
68. Perico L, Benigni A, Remuzzi G. Should COVID-19 Concern Nephrologists? Why and to what extent? The emerging impasse of angiotensin blockade. *Nephron*. (2020) 144:1–9. doi: 10.1159/000507305
69. Cesari M, Sloane PD, Zimmerman S. The controversial condition of cognitive frailty: what it is, what it should be. *J Am Med Dir Assoc.* (2020) 21:146–8. doi: 10.1016/j.jamda.2019.12.013
70. Arentz M, Yim E, Klaff L, Lokhandwala S, Riedo FX, Chong M, et al. Characteristics and outcomes of 21 critically Ill patients with COVID-19 in Washington State. *JAMA.* (2020) 323:1612–4. doi: 10.1001/jama.2020.4326
71. Yang XH, Sun RH, Chen DC. [Diagnosis and treatment of COVID-19: acute kidney injury cannot be ignored]. *Zhonghua Yi Xue Za Zhi.* (2020) 100:1205–8. doi: 10.3760/cma.j.cn112137-20200229-00520
72. Mao L, Jin H, Wang M, Hu Y, Chen S, He Q, et al. Neurologic manifestations of hospitalized patients with coronavirus disease 2019 in Wuhan, China. *JAMA Neurol.* (2020) 77:1–9. doi: 10.1001/jamaneurol.2020.1127
73. Wang HY, Li XL, Yan ZR, Sun XP, Han J, Zhang BW. Potential neurological symptoms of COVID-19. *Ther Adv Neurol Disord.* (2020) 13:1756286420917830. doi: 10.1177/1756286420917830
74. Helms J, Kremer S, Merdji H, Clere-Jehl R, Schenck M, Kummerlen C, et al. Neurologic features in severe SARS-CoV-2 infection. *N Engl J Med.* (2020) 382:2268–70. doi: 10.1056/NEJMc2008597
75. Wu Y, Xu X, Chen Z, Duan J, Hashimoto K, Yang L, et al. Nervous system involvement after infection with COVID-19 and other coronaviruses. *Brain Behav Immun.* (2020) 87:18–22. doi: 10.1016/j.bbi.2020.03.031
76. Moriguchi T, Harii N, Goto J, Harada D, Sugawara H, Takamino J, et al. A first case of meningitis/encephalitis associated with SARS-Coronavirus-2. *Int J Infect Dis.* (2020) 94:55–8. doi: 10.1016/j.ijid.2020.03.062

77. Li Y, Li M, Wang M, Zhou Y, Chang J, Xian Y, et al. Acute cerebrovascular disease following COVID-19: a single center, retrospective, observational study. *Stroke Vasc Neurol.* (2020). doi: 10.2139/ssrn.3550025. [Epub ahead of print].
78. Poyiadji N, Shahin G, Noujaim D, Stone M, Patel S, Griffith B. COVID-19-associated acute hemorrhagic necrotizing encephalopathy: CT and MRI features. *Radiology*. (2020) 296:E119–20. doi: 10.1148/radiol.2020201187
79. Pleasure SJ, Green AJ, Josephson SA. The spectrum of neurologic disease in the severe acute respiratory syndrome coronavirus 2 pandemic infection: neurologists move to the frontlines. *JAMA Neurol.* (2020). doi: 10.1001/jamaneurol.2020.1065. [Epub ahead of print].
80. Cui Y, Tian M, Huang D, Wang X, Huang Y, Fan L, et al. A 55-day-old female infant infected with COVID 19: presenting with pneumonia, liver injury, and heart damage. *J Infect Dis.* (2020) 221:1775–81. doi: 10.1093/infdis/jiaa113
81. Villalba NL, Maouche Y, Ortiz MBA, Sosa ZC, Chahbazian JB, Syrovatkova A, et al. Anosmia and dysgeusia in the absence of other respiratory diseases: should COVID-19 infection be considered? *Eur J Case Rep Intern Med.* (2020) 7:001641. doi: 10.12890/2020_001641
82. Moein ST, Hashemian SM, Mansourafshar B, Khorram-Tousi A, Tabarsi P, Doty RL. Smell dysfunction: a biomarker for COVID-19. *Int Forum Allergy Rhinol.* (2020). doi: 10.1002/alr.22587. [Epub ahead of print].
83. Lechien JR, Chiesa-Estomba CM, De Siati DR, Horoi M, Le Bon SD, Rodriguez A, et al. Olfactory and gustatory dysfunctions as a clinical presentation of mild-to-moderate forms of the coronavirus disease (COVID-19): a multicenter European study. *Eur Arch Otorhinolaryngol.* (2020) 277:2251–61. doi: 10.1007/s00405-020-05965-1
84. Salata C, Calistri A, Parolin C, Palu G. Coronaviruses: a paradigm of new emerging zoonotic diseases. *Pathog Dis.* (2019) 77:ftaa006. doi: 10.1093/femspd/ftaa006
85. Hok K. Morbidity, mortality and coronavirus antigen in previously coronavirus free kittens placed in two catteries with feline infectious peritonitis. *Acta Vet Scand.* (1993) 34:203–10.
86. Doherty MJ. Ocular manifestations of feline infectious peritonitis. *J Am Vet Med Assoc.* (1971) 159:417–24.
87. World Health Organization. *Case Definitions for Surveillance of Severe Acute Respiratory Syndrome (SARS)*.
88. Loon SC, Teoh SC, Oon LL, Se-Thoe SY, Ling AE, Leo YS, et al. The severe acute respiratory syndrome coronavirus in tears. *Br J Ophthalmol.* (2004) 88:861–3. doi: 10.1136/bjo.2003.035931
89. van der Hoek L, Pyrc K, Jebbink MF, Vermeulen-Oost W, Berkhout RJ, Wolthers KC, et al. Identification of a new human coronavirus. *Nat Med.* (2004) 10:368–73. doi: 10.1038/nm1024
90. Vabret A, Mourez T, Dina J, van der Hoek L, Gouarin S, Petitjean J, et al. Human coronavirus NL63, France. *Emerg Infect Dis.* (2005) 11:1225–9. doi: 10.3201/eid1108.050110
91. Qing H, Li Z, Yang Z, Shi M, Huang Z, Song J, et al. The possibility of COVID-19 transmission from eye to nose. *Acta Ophthalmol.* (2020) 98:e388. doi: 10.1111/aos.14412
92. Recalcati S. Cutaneous manifestations in COVID-19: a first perspective. *J Eur Acad Dermatol Venereol.* (2020) 34:e212–3. doi: 10.1111/jdv.16387
93. Epidemiology Working Group for Ncip Epidemic Response CCfDC, Prevention. [The epidemiological characteristics of an outbreak of 2019 novel coronavirus diseases (COVID-19) in China]. *Zhonghua Liu Xing Bing Xue Za Zhi.* (2020). 41:145–51. doi: 10.3760/cma.j.issn.0254-6450.2020.02.003
94. Nishiura H, Kobayashi T, Miyama T, Suzuki A, Jung SM, Hayashi K, et al. Estimation of the asymptomatic ratio of novel coronavirus infections (COVID-19). *Int J Infect Dis.* (2020) 94:154–5. doi: 10.1016/j.ijid.2020.03.020
95. Mizumoto K, Kagaya K, Zarebski A, Chowell G. Estimating the asymptomatic proportion of coronavirus disease 2019. (COVID-19) cases on board the Diamond Princess cruise ship, Yokohama, Japan, 2020. *Euro Surveill.* (2020). 25:2000180. doi: 10.2807/1560-7917.ES.2020.25.10.2000180
96. Ki M, Task Force for -nCo V. Epidemiologic characteristics of early cases with 2019 novel coronavirus (2019-nCoV) disease in Korea. *Epidemiol Health.* (2020) 42:e2020007. doi: 10.4178/epih.e2020007
97. Lu X, Zhang L, Du H, Zhang J, Li YY, Qu J, et al. SARS-CoV-2 Infection in Children. *N Engl J Med.* (2020) 382:1663–5. doi: 10.1056/NEJMc2005073
98. Kimball A, Hatfield KM, Arons M, James A, Taylor J, Spicer K, et al. Asymptomatic and presymptomatic SARS-CoV-2 infections in residents of a long-term care skilled nursing facility - king county, Washington, March 2020. *MMWR Morb Mortal Wkly Rep.* (2020) 69:377–81. doi: 10.15585/mmwr.mm6913e1
99. Hu Z, Song C, Xu C, Jin G, Chen Y, Xu X, et al. Clinical characteristics of 24 asymptomatic infections with COVID-19 screened among close contacts in Nanjing, China. *Sci China Life Sci.* (2020) 63:706–11. doi: 10.1007/s11427-020-1661-4
100. Park J, Lee DS, Christakis NA, Barabasi AL. The impact of cellular networks on disease comorbidity. *Mol Syst Biol.* (2009) 5:262. doi: 10.1038/msb.2009.16
101. Fowler RA, Lapinsky SE, Hallett D, Detsky AS, Sibbald WJ, Slutsky AS, et al. Critically ill patients with severe acute respiratory syndrome. *JAMA.* (2003) 290:367–73. doi: 10.1001/jama.290.3.367
102. Chan JW, Ng CK, Chan YH, Mok TY, Lee S, Chu SY, et al. Short term outcome and risk factors for adverse clinical outcomes in adults with severe acute respiratory syndrome (SARS). *Thorax.* (2003) 58:686–9. doi: 10.1136/thorax.58.8.686
103. Booth CM, Matukas LM, Tomlinson GA, Rachlis AR, Rose DB, Dwosh HA, et al. Clinical features and short-term outcomes of 144 patients with SARS in the greater Toronto area. *JAMA.* (2003) 289:2801–9. doi: 10.1001/jama.289.21.JOC30885
104. Assiri A, Al-Tawfiq JA, Al-Rabeeah AA, Al-Rabiah FA, Al-Hajjar S, Al-Barrak A, et al. Epidemiological, demographic, and clinical characteristics of 47 cases of Middle East respiratory syndrome coronavirus disease from Saudi Arabia: a descriptive study. *Lancet Infect Dis.* (2013) 13:752–61. doi: 10.1016/S1473-3099(13)70204-4
105. Yang YM, Hsu CY, Lai CC, Yen MF, Wikramaratna PS, Chen HH, et al. Impact of Comorbidity on Fatality Rate of Patients with Middle East Respiratory Syndrome. *Sci Rep.* (2017) 7:11307. doi: 10.1038/s41598-017-10402-1
106. Wang B, Li R, Lu Z, Huang Y. Does comorbidity increase the risk of patients with COVID-19: evidence from meta-analysis. *Aging.* (2020) 12:6049–57. doi: 10.18632/aging.103000
107. Jain V, Yuan JM. Predictive symptoms and comorbidities for severe COVID-19 and intensive care unit admission: a systematic review and meta-analysis. *Int J Public Health.* (2020) 65:533–46. doi: 10.1007/s00038-020-01390-7

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Public Health

ORIGINAL RESEARCH
published: 13 August 2020
doi: 10.3389/fpubh.2020.00473

Modeling the Onset of Symptoms of COVID-19

Joseph R. Larsen[1,2], *Margaret R. Martin*[3], *John D. Martin*[4], *Peter Kuhn*[2*] and *James B. Hicks*[2*]

[1] Quantitative and Computational Biology, Department of Biological Science, University of Southern California, Los Angeles, CA, United States, [2] USC Michelson Center for Convergent Bioscience, University of Southern California, Los Angeles, CA, United States, [3] Nexus Development PA LLC, Redwood City, CA, United States, [4] NanoCarrier Co., Ltd., Chiba, Japan

COVID-19 is a pandemic viral disease with catastrophic global impact. This disease is more contagious than influenza such that cluster outbreaks occur frequently. If patients with symptoms quickly underwent testing and contact tracing, these outbreaks could be contained. Unfortunately, COVID-19 patients have symptoms similar to other common illnesses. Here, we hypothesize the order of symptom occurrence could help patients and medical professionals more quickly distinguish COVID-19 from other respiratory diseases, yet such essential information is largely unavailable. To this end, we apply a Markov Process to a graded partially ordered set based on clinical observations of COVID-19 cases to ascertain the most likely order of discernible symptoms (i.e., fever, cough, nausea/vomiting, and diarrhea) in COVID-19 patients. We then compared the progression of these symptoms in COVID-19 to other respiratory diseases, such as influenza, SARS, and MERS, to observe if the diseases present differently. Our model predicts that influenza initiates with cough, whereas COVID-19 like other coronavirus-related diseases initiates with fever. However, COVID-19 differs from SARS and MERS in the order of gastrointestinal symptoms. Our results support the notion that fever should be used to screen for entry into facilities as regions begin to reopen after the outbreak of Spring 2020. Additionally, our findings suggest that good clinical practice should involve recording the order of symptom occurrence in COVID-19 and other diseases. If such a systemic clinical practice had been standard since ancient diseases, perhaps the transition from local outbreak to pandemic could have been avoided.

Keywords: COVID-19, Markov, probability, symptoms, stochastic, model, disease, influenza

OPEN ACCESS

Edited by:
Alexander Rodriguez-Palacios, Department of Medicine, Case Western Reserve University, United States

Reviewed by:
Herve Seligmann, The Hebrew University of Jerusalem, Israel
Tauqeer Hussain Mallhi, Department of Clinical Pharmacy, College of Pharmacy, Al Jouf University, Saudi Arabia

***Correspondence:**
Peter Kuhn
pkuhn@usc.edu
James B. Hicks
jameshic@usc.edu

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Public Health

Received: *13 April 2020*
Accepted: *27 July 2020*
Published: *13 August 2020*

Citation:
Larsen JR, Martin MR, Martin JD, Kuhn P and Hicks JB (2020) Modeling the Onset of Symptoms of COVID-19. Front. Public Health 8:473. doi: 10.3389/fpubh.2020.00473

INTRODUCTION

The current pandemic of Coronavirus Disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has undergone an observed exponential increase of cases that has overrun hospitals across the world (1). Many people have mild forms of the disease and are advised not to go to the hospital or to seek a diagnostic test because they can recover at home. A large number of others are asymptomatic (2). Infected individuals are highly contagious and can transmit the disease even if they are asymptomatic, and this fact furthers the need to isolate and test often (2). In addition, COVID-19 is two to three times more contagious than influenza (3). Due to these characteristics, outbreaks of COVID-19 occur in clusters (4). Identifying COVID-19 early could reduce the number and size of clusters, but early symptoms are not well-defined. The Center for Disease Control and Prevention (CDC) in the USA and the World

Health Organization (WHO) currently advise the public to call their doctor if they believe they have been exposed to COVID-19 or exhibit fever and cough (5). However, fever and cough are associated with other respiratory diseases such as influenza (6–8). Influenza, with an estimated number of symptomatic cases in the millions annually in the U.S. alone (9), also is commonly associated with fever and cough (6). Similarly to COVID-19, the Middle East Respiratory Syndrome (MERS) and the Severe Acute Respiratory Syndrome (SARS) are respiratory illnesses contracted from coronaviruses called the MERS-Related Coronavirus (MERS-CoV) and SARS-Related Coronavirus (SARS-CoV), respectively (7). The symptoms of these diseases also overlap with COVID-19. The capacity to discern differences in these common symptoms, such as order of occurrence and likely first symptoms, would aid in early recognition. If health care workers recorded and published clinically-observed and/or patient-reported sequences of symptoms, the reported data could be evaluated as an additional tool for early recognition of COVID-19 to increase self-surveillance and reduce spread. If such a widespread clinical practice had been instituted in the past, perhaps local outbreaks of influenzas, coronaviruses, and other diseases might have been contained before becoming pandemics.

To this end, we assumed that symptoms and their orders are independent variables and created a model that approximates the probability of symptoms occurring in specific orders using available, non-ordered patient data. The use of these assumptions and data was necessary given the lack of ordered data. To do this, we applied a Markov Process to determine the order of occurrence of common symptoms of respiratory diseases. We have previously used a Markov Chain to predict cancer metastasis location (10–14). A Markov Process is defined as a stochastic sequence of events in which the likelihood of the next state only depends on the current state rather than past or future states (15). In this case, we defined each state to be the specific symptoms that a patient has experienced, and each transition is only dependent on these symptoms. As a result, we can determine the likelihood of each symptom stepwise using a Markov Process. We defined the state probability of a node as the frequency that a patient has a particular combination of symptoms divided by the total number of patients that exhibit the same number of symptoms. The transition probability between two states is defined as the likelihood of acquiring a single specific symptom divided by the likelihood of acquiring all possible next symptoms. We then applied a greedy algorithmic approach using the transition probabilities to calculate the probability of all possible orders toward determining the most and least likely orders of symptoms.

In this study, we first defined this specific application of a Markov Process applied to a graded partially ordered set (poset), which we refer to as the Stochastic Progression Model. In this case, our graded poset represents all possible combinations of symptoms and all possible orders of symptom occurrence. It is graded because the possible combinations of symptoms are ranked by the number of symptoms that they each represent. For example, the symptom combination of fever and cough has the same rank as the combination of cough and diarrhea. We found that the Stochastic Progression Model for adults that are symptomatic indicates that there may be an order of discernible symptoms in COVID-19, but the order of symptoms seems to be independent of severity of the case on admission. From there, we compared the most likely order of symptoms in other respiratory diseases to COVID-19. To expand on our results, we analyzed a larger set of symptoms that are common to all respiratory diseases studied here and sought to decipher further distinctions.

MATERIALS AND METHODS

Data Collection

Patient data from this study was collected from various reports in literature on the frequencies of symptoms in COVID-19, influenza, MERS, and SARS (**Supplemental Tables 1, 2**). Each dataset was used either to approximate order of symptoms, to confirm our results, or to analyze first symptoms in COVID-19 or influenza. For all of these applications, we used the reported patient data to simulate patients with various combinations of symptoms experienced and then applied the simulated data to perform the analyses.

The main dataset of COVID-19 patients of the World Health Organization, containing 55,924 confirmed cases, was obtained through review of national and local governmental reports and observations made during visits to areas with infected individuals in China that occurred from February 16 to 24, 2020 (8). A confirmation dataset of COVID-19 patients, containing 1,099 confirmed cases, was obtained by the China Medical Treatment Expert Group for COVID-19 from medical records and other compiled data of hospitalized patients and outpatients that were diagnosed with COVID-19. This data was reported to the National Health Commission of China from December 11, 2019 to January 29, 2020 (16). For both COVID-19 datasets, myalgia was reported as myalgia or arthralgia. We assumed that most patients with myalgia also had arthralgia, and therefore we used the frequency of myalgia or arthralgia as a frequency for myalgia when simulating data. The influenza dataset, containing 2,470 confirmed cases, was collected by researchers at the University of Michigan from a retrospective pooled analysis of mostly unvaccinated patients participating in phase 2 and 3 clinical trials that were conducted in North America, Europe, and the Southern Hemisphere from 1994 to 1998 (6). This group of patients has a mean age of 35 and each exhibited multiple symptoms. Vomiting and diarrhea were not reported in this influenza dataset, but they are common among respiratory disease. Although adult patients at times may experience vomiting and diarrhea when infected with influenza, these symptoms are rare (17). Therefore, we approximate the frequency of these symptoms as 0.010 in this case. The datasets representing symptom frequency in MERS, containing 245 patients, and SARS, containing 357 patients, were collected on admission and were reported as clinical data from physicians, Dr. Yin, at the Beijing Chao-Yang Hospital and Dr. Wunderink, at the Northwestern University Feinberg School of Medicine (7). The patients included in these datasets varied in age and pre-existing conditions. In the cases of SARS, the patients tended to be younger and have fewer pre-existing conditions than in the cases of MERS.

We used initial frequency data of MERS and SARS to further ascertain early symptoms of disease. The MERS initial symptom frequency dataset, containing 45 confirmed cases, was collected from electronic medical records at the Samsung Medical Center in Seoul, South Korea that contained onset symptom data about patients in the 2015 Korean MERS outbreak (**Supplemental Table 3**) (18). The SARS initial symptom frequency dataset, containing 144 confirmed cases, was collected from hospital records including information of early symptoms in patients dating from March 7 to April 10, 2003 during an outbreak in the greater Toronto area (**Supplemental Table 4**) (19).

Lastly, two additional datasets were collected to determine the utility of using first symptoms as early indicators of COVID-19 and influenza. The COVID-19 dataset used, containing 138 patients, was independent of all prior COVID-19 datasets. This data was obtained from electronic medical records of patients admitted to the Zhongnan Hospital of Wuhan University from January 1 to 28, 2020 (20). The symptom data was collected at onset of disease and all patients experienced pneumonia due to COVID-19. In this dataset, nausea and vomiting were reported separately for COVID-19. We assumed that most patients who experience vomiting, which is reported with a frequency of 0.036, also experience nausea, which is reported with a frequency of 0.101, and therefore to simulate the data, we defined the frequency for nausea/vomiting as 0.101. The influenza dataset used reported 20 confirmed cases of influenza and 400 confirmed negative cases of influenza and is independent from any other influenza dataset we used (21). The symptom data was collected through questionnaires and observations by medical professionals during the influenza seasons of 2006 and 2007 of infected patients admitted at the Department of Internal Medicine and Infectious Diseases and the Department of Pulmonology at the University Medical Center Utrecht. Like the other influenza dataset described above, vomiting and diarrhea were not reported in this dataset. So, we once again assumed the frequency of these symptoms to be 0.010 (17). Because this study was conducted in 2006 and 2007, prior to the COVID-19 outbreak, we assumed these patients were negative for COVID-19 as well. So, this 400-patient group was used as the dataset that represents individuals negative for both COVID-19 and influenza (**Supplemental Table 5**).

Simulating Symptom Progression From Patient Data

The Stochastic Progression Model was built in R under version 3.5.2 and was illustrated by using the hasse function in the hasseDiagram_0.1.3 library (code available online: https://github.com/j-larsen/Stochastic_Progression_of_COVID-19_Symptoms) (22, 23). Each respiratory disease report was represented by a corresponding data frame, with columns as symptoms, one row as the frequency of the symptoms observed in the study, and the other row as the frequency multiplied by 1,000. The multiple of the frequency is defined as the frequency count, which represents the probability of a symptom in a theoretical sample size of 1,000 simulated patients. Additionally, the state of an individual is displayed through a character array of ones and zeros, where ones represent the presence of a symptom and zeroes represent its absence. This process of simulating a symptom is analogous to a jar of marbles of either two colors. The probability of pulling one color of marble (i.e., a specific symptom) is illustrated by the frequency count because the total number of marbles in the jar is 1,000 and the frequency count for each is the number of the specific color of marbles in the jar.

We then simulated data of 500,000 patients, by randomly selecting if a patient has or does not have a symptom using the procedure described above and storing that information in a data frame that represents patients as rows and symptoms as columns. We assumed the occurrence of symptoms are random and independent. Considering these assumptions, we built the character arrays by applying the jar of marbles method for each simulated patient. The method repeats for each patient and involves pulling a marble from a series of jars representing each symptom. The information from each randomly pulled marble is stored in the corresponding cell of the character array in the correct column representing the symptom and the row representing the simulated patient. This process is repeated for all 500,000 simulated patients for all symptoms.

Building the Stochastic Progression Model

The Stochastic Progression Model is illustrated as a directed acyclic graph with nodes, representing the power set of Boolean vectors. The power sets of Boolean vectors each represent a possible state of a patient by noting the absence or presence of specific symptoms. The edges, which illustrate the transition from one state to another, were selected specifically using key definitions and assumptions to create a poset. We defined the states at the nodes as symptoms that a patient has experienced up until this point. We created and directed edges from states with fewer symptoms to more starting at the minimum set of a Boolean vector of all zeros, which indicates a person with no symptoms. First, we assume that each symptom occurs one at a time, even if the difference in time is infinitesimal. With this assumption, a node can only be directed to other nodes that denote the same set of symptoms plus one additional symptom. Second, we assume that if a patient does not digress and does not die, they will eventually acquire all symptoms reaching the maximum set of a Boolean vector, which represents a patient that has exhibited all symptoms. Applying these assumptions to form the directed acyclic graph creates a Hasse Diagram of a graded poset that follows a Markov Process altogether comprising the Stochastic Progression Model.

Calculating State and Transition Probabilities

The nodes in the Hasse Diagram represent states of a patient by indicating the specific symptoms exhibited, and the edges represent transitions between these states. Therefore, we next needed to apply state probabilities to each node and transition probabilities to the directed edges. First, we labeled each simulated patient by summing the respective Boolean vector to find the number of symptoms for each patient. Then, to get the state probability of each node, we divided the number of

simulated patients that are represented by the current Boolean vector by the total number of patients who have the same number of symptoms. To approximate the transition probability between two nodes (originating and terminating), we divided the number of simulated patients that are represented by the terminating node by the number of simulated patients that are represented by nodes characterized by the same number of symptoms as the terminating node, including the terminating node. The error of each node is determined by the sum of the products of the transition probabilities leading to that node subtracted from the state probability of the node. Then, the error of each implementation of the model was defined as the error of the node with the highest absolute value of error (**Supplemental Figures 2–13**). The transition probabilities signify the likelihoods of transitions from one node to another, and the aggregates of the transition probabilities in a sequence represent the likelihoods of the paths. These paths illustrate the order of symptoms when infected with a respiratory disease by observing the stepwise addition of symptoms when traversing down nodes in the path. The most and least likely paths were determined using a greedy algorithmic approach. This approach consists of selecting local maximum or minimum edges stepwise, which results in a most and least likely path, respectively. If the maximum (or minimum) transition probability from a specific node was within error of other transition probabilities of edges from the same originating node, we grouped the terminating nodes when finding the most (or least) likely path. In these cases, we could not distinguish a difference in likelihood between these specific transitions. The paths create a possible order of symptoms via the poset, each having a specific likelihood of occurrence.

RESULTS

A Possible Order of Discernible Symptoms in COVID-19

The WHO-China Joint Report from February 16 to 24, 2020 includes rates of symptom occurrence at presentation from 55,924 confirmed cases of COVID-19 (8). We identified symptoms that were easily discernible or objective (i.e., fever, cough, diarrhea, and nausea/vomiting) in comparison to other reported symptoms, such as inflammations of blood vessel epithelia (24), neurological effects (25), and rash-like symptoms (26). These symptoms are also common in other respiratory diseases. Thus, we chose to implement these four symptoms in the Stochastic Progression Model (**Supplemental Table 1**). To confirm the validity of the model, we first determined the possible sequences of symptom occurrence when the probabilities are uniformly random for each symptom. In addition to all possible orders of occurrence of the four symptoms, the diagram displays the most and least likely paths of the four symptoms, depicted by red lines and blue lines, respectively (**Figures 1A,B**). The most and least likely paths describe the most and least likely series of symptoms that a random infected person from the population in the dataset may experience. In this case, each possible path is equally likely, with no path having any higher probability than any other.

We then created another implementation of the Stochastic Progression Model and utilized the data in the WHO-China Joint Report (COVID-19 with $N = 55{,}924$) (8). With this implementation, we determined the most and least likely paths (**Figure 1C**). In this case, a person infected with COVID-19 is most likely to experience symptoms in the order of fever, cough, nausea/vomiting, then diarrhea (**Figure 1D**). The least likely path starts at diarrhea and nausea/vomiting and is followed by cough, and finally fever (**Figure 1E**). We confirmed these results with a smaller dataset (COVID-19 with $N = 1{,}099$) (**Figures 1D,E**, and **Supplemental Figure 1**) (16). The likelihoods of transitioning to fever, 0.769, and then to cough, 0.958, are high, and these observations indicate that a large portion of infected symptomatic patients may follow this path. Finally, this implementation of the model predicts that nausea/vomiting occurs before diarrhea. These two results suggest that in patients with SARS-CoV-2, the body first develops fever, then upper respiratory symptoms and finally symptoms of the upper then lower gastrointestinal (GI) tract.

To further investigate these symptom paths, we implemented the Stochastic Progression Model with the main dataset (COVID-19 with $N = 55{,}924$) (8), to determine the likely downstream paths when the first one or two symptoms are forced to a certain state (**Figures 1F–I**). The gray lines represent the "forced" paths. The rest of the paths were determined as before with a greedy algorithmic approach. We found that the most likely orders of the downstream path are consistent with the most likely orders of the unforced paths. Even if the first symptom is forced to be an unlikely one (e.g., diarrhea), the downstream paths maintain the most likely order of the other three symptoms that we originally determined (**Figure 1F**). Similarly, the GI tract effects occur first in the forced least likely paths (**Figure 1G**). When forcing the path one step further by predetermining the first two symptoms for both the most and least likely paths, the findings remain the same (**Figures 1H,I**).

Order of Discernible Symptoms in COVID-19 Is Independent of Severity of Disease on Admission

The confirmation dataset of COVID-19 cases ($N = 1{,}099$) separates the reported 1,099 cases between severe and non-severe patients as designated on admission (16). To investigate the effects of severity on the order of discernible symptoms, we implemented each set of cases separately using the Stochastic Progression Model. We found that the most and least likely paths are identical in severe and non-severe cases and to our original findings above (**Figure 2**). To illustrate the similarities, the largest difference in likelihood is observed when transitioning from no symptoms to fever in the most likely path. In severe and non-severe cases, the probability is 0.775 and 0.818, respectively, indicating a difference of 0.043. These results suggest that severity does not affect the order of discernible symptoms, and they are consistent with the hypothesis of fever as the first symptom of COVID-19.

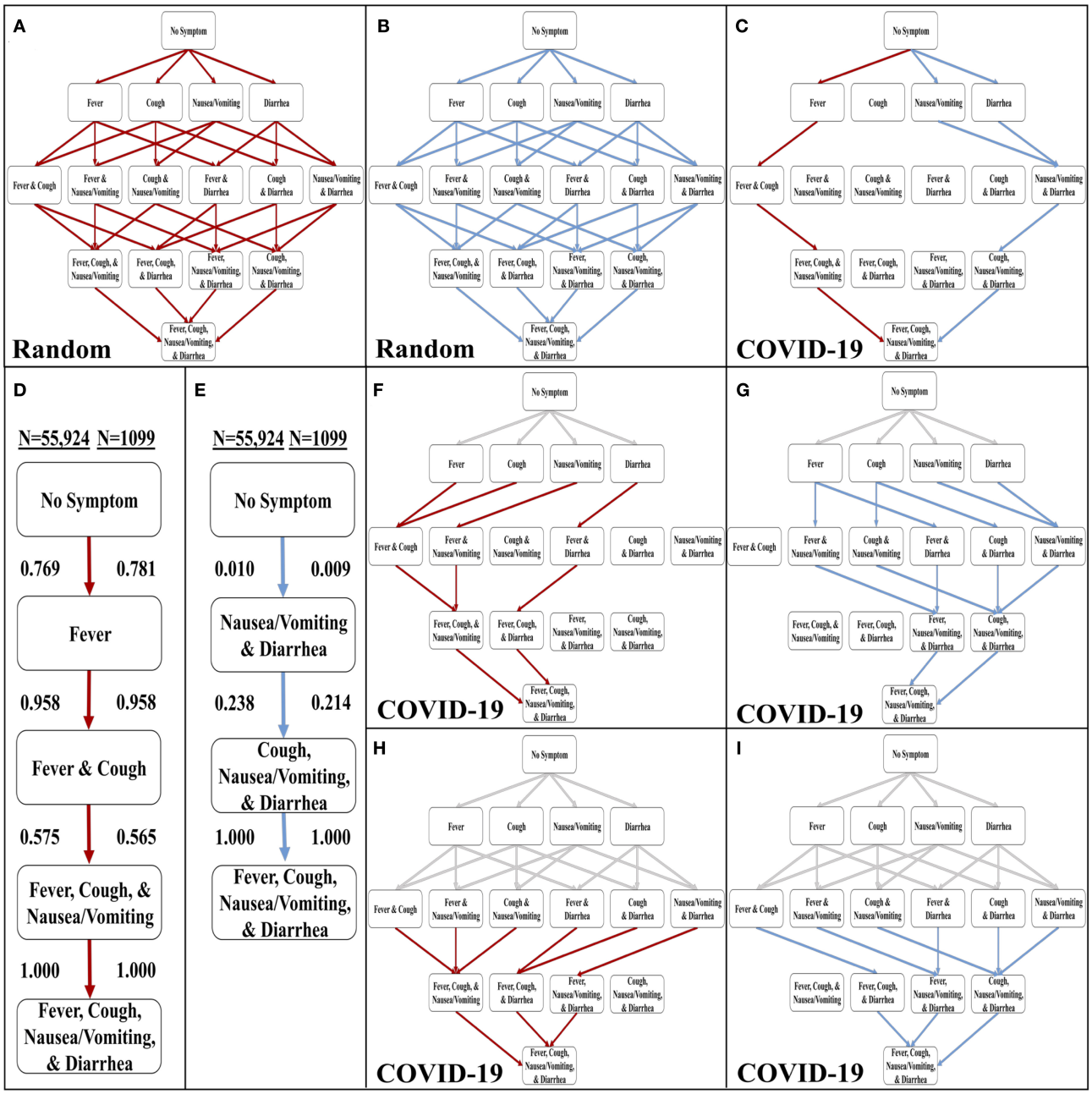


FIGURE 1 | Development of the stochastic progression model for COVID-19. **(A)** The most likely paths (red) in the Hasse Diagram for symptoms with random likelihoods of occurring. **(B)** The least likely paths (blue) in the Hasse Diagram for symptoms with random likelihoods of occurring. **(C)** The most likely (red) and least likely (blue) paths in the Hasse Diagram for symptoms in COVID-19. **(D)** The most likely order of symptoms in COVID-19 based on our Stochastic Progression Model determined from transition probabilities presented here. **(E)** The least likely order of symptoms in COVID-19 based on our Stochastic Progression Model determined from transition probabilities presented here. **(F)** Hasse Diagram of the most likely paths (red) after traveling any forced path (gray) of patients with one symptom. **(G)** Hasse Diagram of the least likely paths (blue) after traveling any forced path (gray) of patients with one symptom. **(H)** Hasse Diagram of the most likely paths (red) after traveling any forced path (gray) of patients with two symptoms. **(I)** Hasse Diagram of the least likely paths (blue) after traveling any forced path (gray) of patients with two symptoms.

Variation of Order of Discernible Symptoms Between Respiratory Diseases

The four discernible symptoms are objective and relatively easy for patients and clinicians to confirm. So, we developed implementations of the Stochastic Progression Model using these symptoms to determine the most likely and least likely paths for four respiratory diseases: COVID-19, influenza, MERS, and SARS (**Figures 3A–D**) (6–8). The most likely order of occurrence

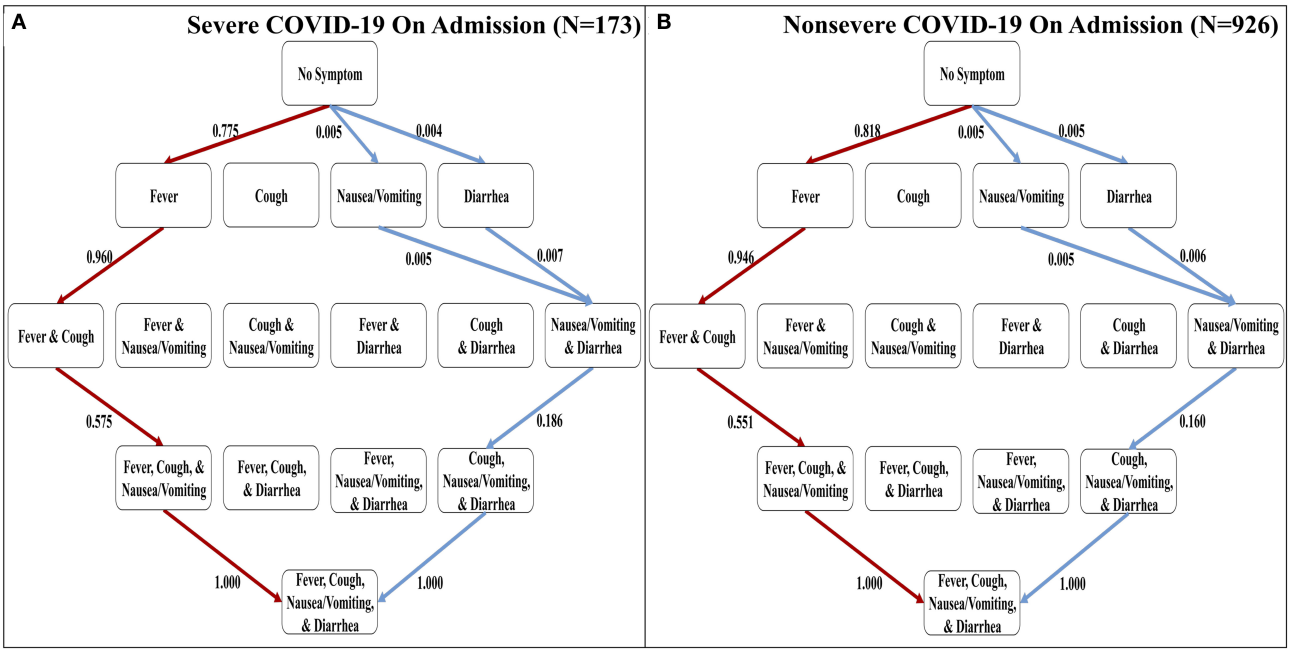


FIGURE 2 | The most and least likely paths of discernible symptoms in severe and non-severe COVID-19 cases on admission. **(A)** Hasse Diagram of the most likely paths (red) and least likely paths (blue) in COVID-19 for cases designated as severe on admission determined from transition probabilities presented here. **(B)** Hasse Diagram of the most likely paths (red) and least likely paths (blue) in COVID-19 for cases designated as non-severe on admission determined from transition probabilities presented here.

of symptoms in COVID-19 is fever, cough, nausea/vomiting, and diarrhea (**Figure 3A**). This path is identical to influenza except the order of the initial two symptoms is switched (**Figure 3B**). On the other hand, the predicted most likely paths (i.e., fever, cough, diarrhea, and then nausea/vomiting) are the same for MERS and SARS (**Figures 3C,D**). This order has one difference from the most likely path in COVID-19 in that the order of the final two symptoms are reversed. The least likely path of MERS starts with either nausea/vomiting or diarrhea as the first step. These steps are followed by cough, and finally fever. In contrast, the least likely path of SARS is cough, nausea/vomiting, and diarrhea in any order, and then finally fever. However, the least likely path of symptoms in COVID-19 is the same as the least likely path in MERS, and the least likely path of influenza is unique compared to the other diseases. It is not detectable whether nausea/vomiting or diarrhea are the first symptoms in influenza, but after these two, the least likely path continues from there to fever then cough. This observation further illustrates the strong link of cough to influenza. As for coronavirus-related diseases, the strongest first indicator is fever followed by cough.

Comparing the Order of Most Common Symptoms in Respiratory Diseases With COVID-19

Although active surveillance of the order discernible symptoms (i.e., fever, cough, nausea/vomiting, and diarrhea) could be useful due to the distinctive most and least likely paths that we determined, we expanded our analysis to the seven symptoms commonly observed in all four respiratory diseases studied here. So, we created a second set of symptoms that amends sore throat, myalgia, and headache to the original set of symptoms (**Supplemental Table 2**). The three additional symptoms are more subjective (6–8). The seven-symptom implementation of the Stochastic Progression Model of COVID-19 shows that these additional symptoms did not perturb our initial ordering of fever, coughing, nausea/vomiting, and diarrhea, but instead added another level of intricacy in the middle of the likely paths (**Figure 4**). We still find that the most likely path first transitions to fever, indicating that fever is the most likely first symptom. From there, the most likely next symptom is cough once again. Then, we observe an undetectable difference in likelihood of transitioning to either sore throat, headache, or myalgia, indicating that all three are likely to occur next before proceeding. The final two nodes are consistent with the four-symptom order by indicating that nausea/vomiting then diarrhea occur last. Although this implementation is more complex because it has seven symptoms, it is consistent with our earlier findings. The most likely path of COVID-19 symptoms is fever, then cough, and next either sore throat, myalgia, or headache, followed by nausea/vomiting, and finally diarrhea, and this order is the same as the one indicated by the implementation developed from the confirmation dataset (COVID-19 with $N = 1{,}099$) (**Figure 4**) (16).

We also implemented the Stochastic Progression Model with the same seven symptoms in influenza, SARS, and MERS

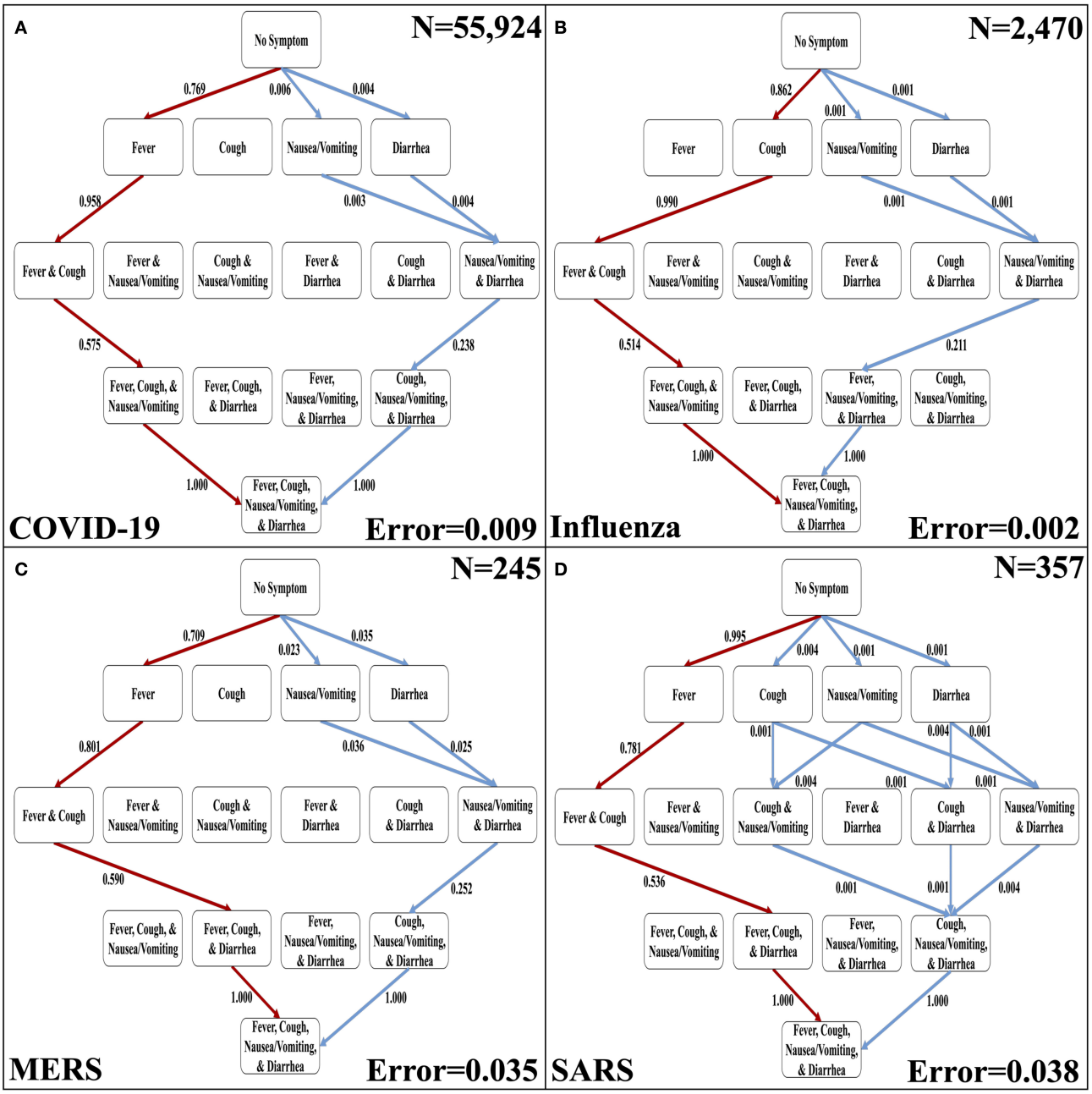


FIGURE 3 | The most likely and least likely paths of discernible symptoms in respiratory diseases. **(A)** The most likely paths (red) and least likely paths (blue) in a Hasse Diagram for COVID-19 symptoms. **(B)** The most likely paths (red) and least likely paths (blue) in a Hasse Diagram for influenza symptoms. **(C)** The most likely paths (red) and least likely paths (blue) in a Hasse Diagram for MERS symptoms. **(D)** The most likely paths (red) and least likely paths (blue) in a Hasse Diagram for SARS symptoms. For each diagram, the most and least likely paths are determined from the transition probabilities that are depicted on the edges. Additionally, error of transition probabilities and sample size (*N*) are presented.

datasets to compare and contrast disease progression with that in COVID-19 (**Figure 5**) (6–8). The results for influenza indicate that cough or myalgia may occur first (**Figure 5A**). After these two symptoms occur, the order of symptoms is headache, sore throat and fever. Finally, vomiting/nausea and diarrhea have an undetectable difference in probability of occurring last. The MERS implementation displays a most likely path in which fever will occur first, followed by cough, headache, and then myalgia (**Figure 5B**). These are followed by an undetectable difference in likelihood of headache and diarrhea occurring. Finally, sore throat and nausea/vomiting will occur last with an undetectable difference. The implementation for SARS shows

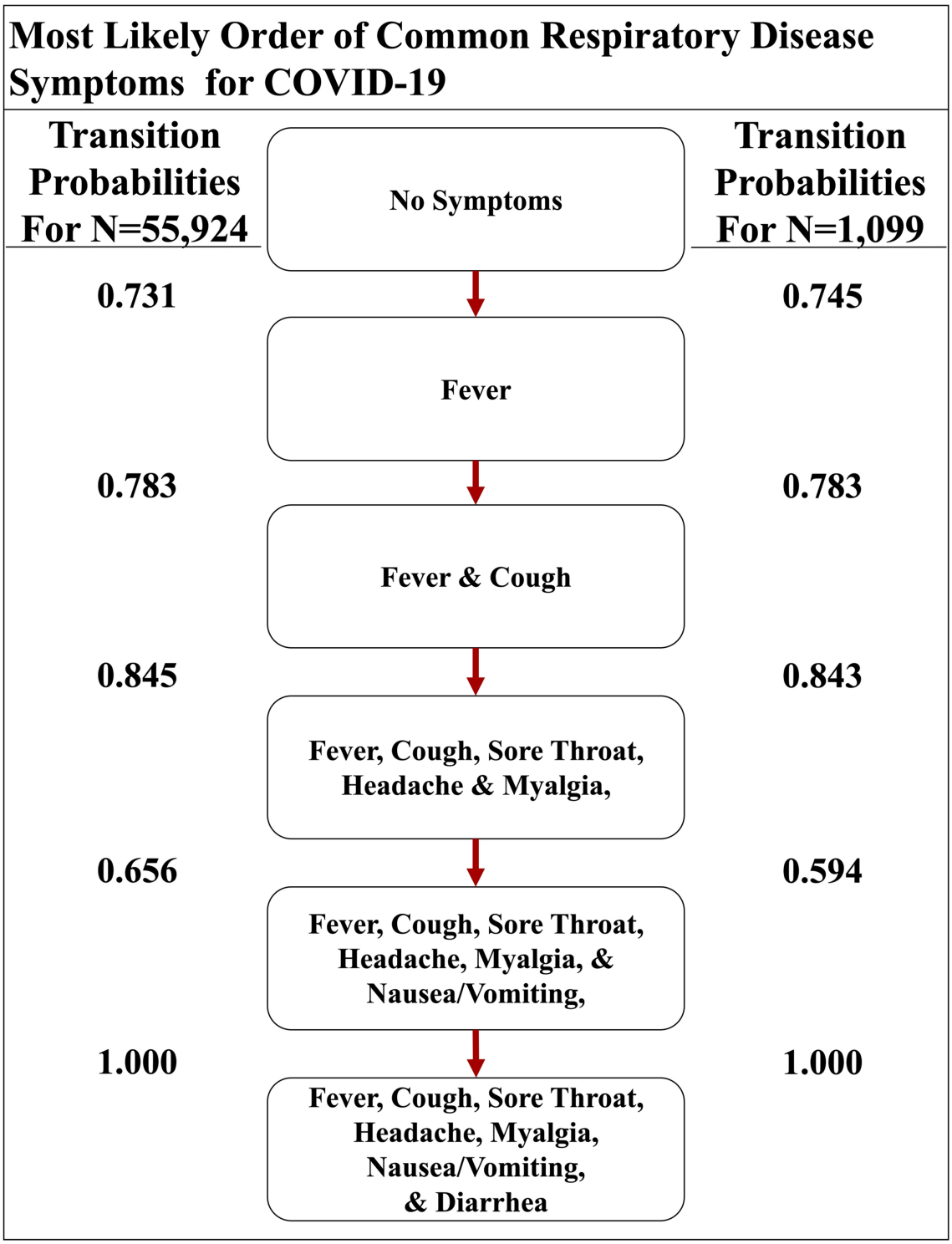


FIGURE 4 | The most likely path of common respiratory symptoms in COVID-19. The most likely path of seven common symptoms of COVID-19, determined by the transition probabilities that are also listed between nodes, of two datasets here.

that fever is most likely to occur first, followed by an undetectable difference in transition probability of cough and myalgia, which is similar to the other coronavirus-related diseases (**Figure 5C**). Next, headache is most likely. Finally, diarrhea, sore throat and nausea/vomiting occur with an undetectable difference in likelihood.

To illustrate the uniqueness of the most likely path of COVID-19, we found the transition probabilities of the same path in

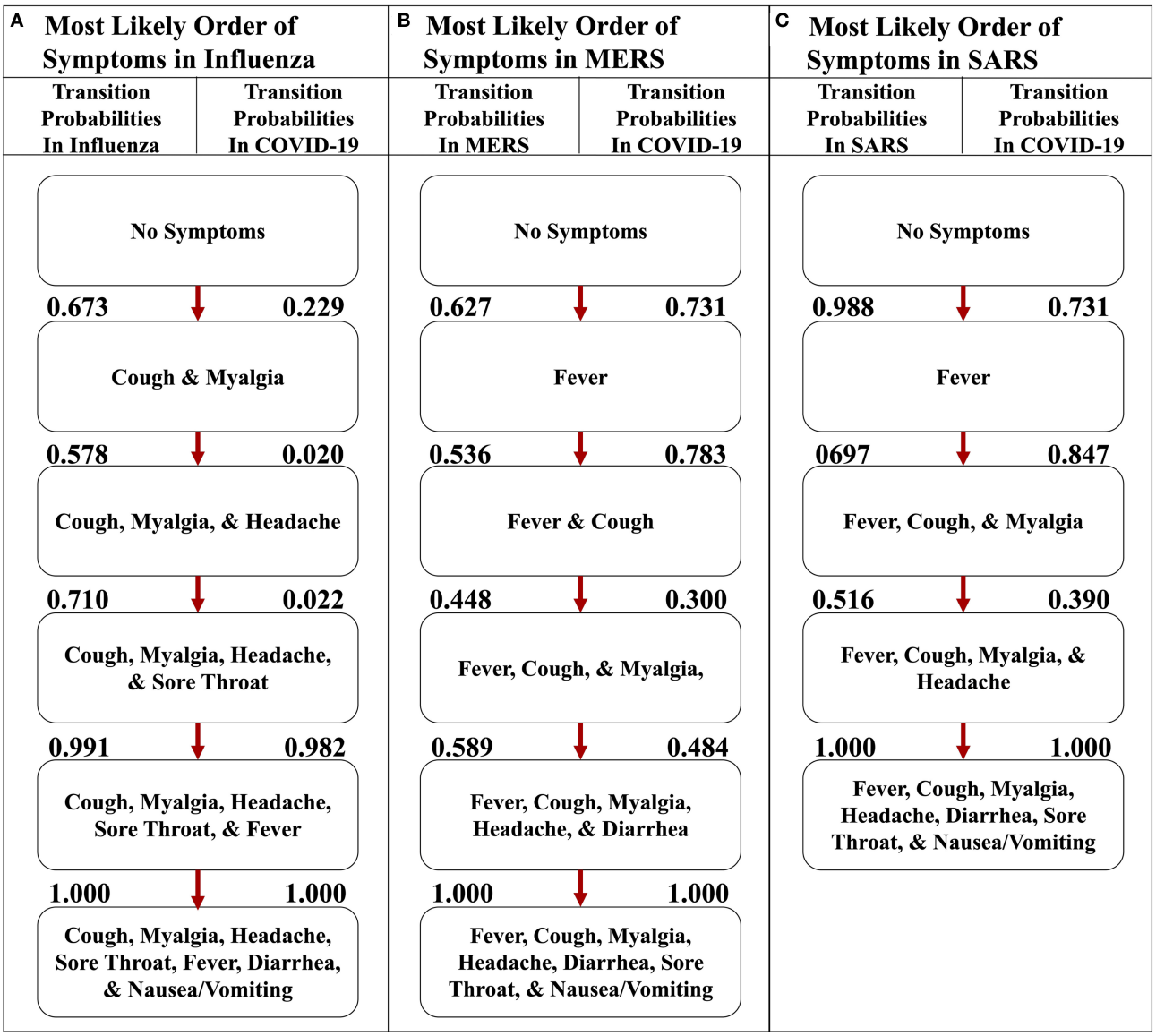


FIGURE 5 | The most likely paths of symptoms in influenza, MERS, and SARS vs. COVID-19. **(A)** The most likely path of seven common symptoms of influenza with the transition probabilities listed between nodes. **(B)** The most likely path of seven common symptoms of MERS with the transition probabilities listed between nodes. **(C)** The most likely path of seven common symptoms of SARS with the transition probabilities listed between nodes. For each path, the transition probabilities in COVID-19 are listed on the right. The most likely paths for each respective disease here are determined from the transition probabilities listed between nodes on the left.

the other respiratory diseases (**Figure 6**). When comparing and contrasting the probabilities, we found that the implementation representing COVID-19 strongly asserts that the first symptom will be fever and cough will soon follow because the transition probabilities are 0.731 and 0.783, respectively (**Figure 6A**), whereas the influenza implementation indicates that fever is very unlikely to occur first with a probability of only 0.035 (**Figure 6B**). Additionally, the implementations of MERS and SARS data also have a high likelihood of transitioning to fever first, with a probability of 0.627 and 0.988, respectively (**Figures 6C,D**). The second symptom of the most likely path of COVID-19 is cough, with a probability of 0.783, but the others do not have a similar high probability. For example, the respiratory disease with the highest probability at that transition is MERS at 0.536. However, after fever and cough, COVID-19 and the other three respiratory diseases have a similarly high likelihood of the three subjective symptoms (i.e., sore throat, headache, and myalgia). Finally, the most likely path of COVID-19 ends with nausea/vomiting and then diarrhea. These observations are consistent with the symptoms described by the CDC and support the notion that fever followed by cough seems highly likely to be diagnosed as COVID-19 (5).

Also, comparing the transition probabilities of paths in the same disease illustrates the significance of the most likely pathways. For example, the lowest transition probability in the most likely path of influenza is 0.578 (**Figure 5A**), whereas when

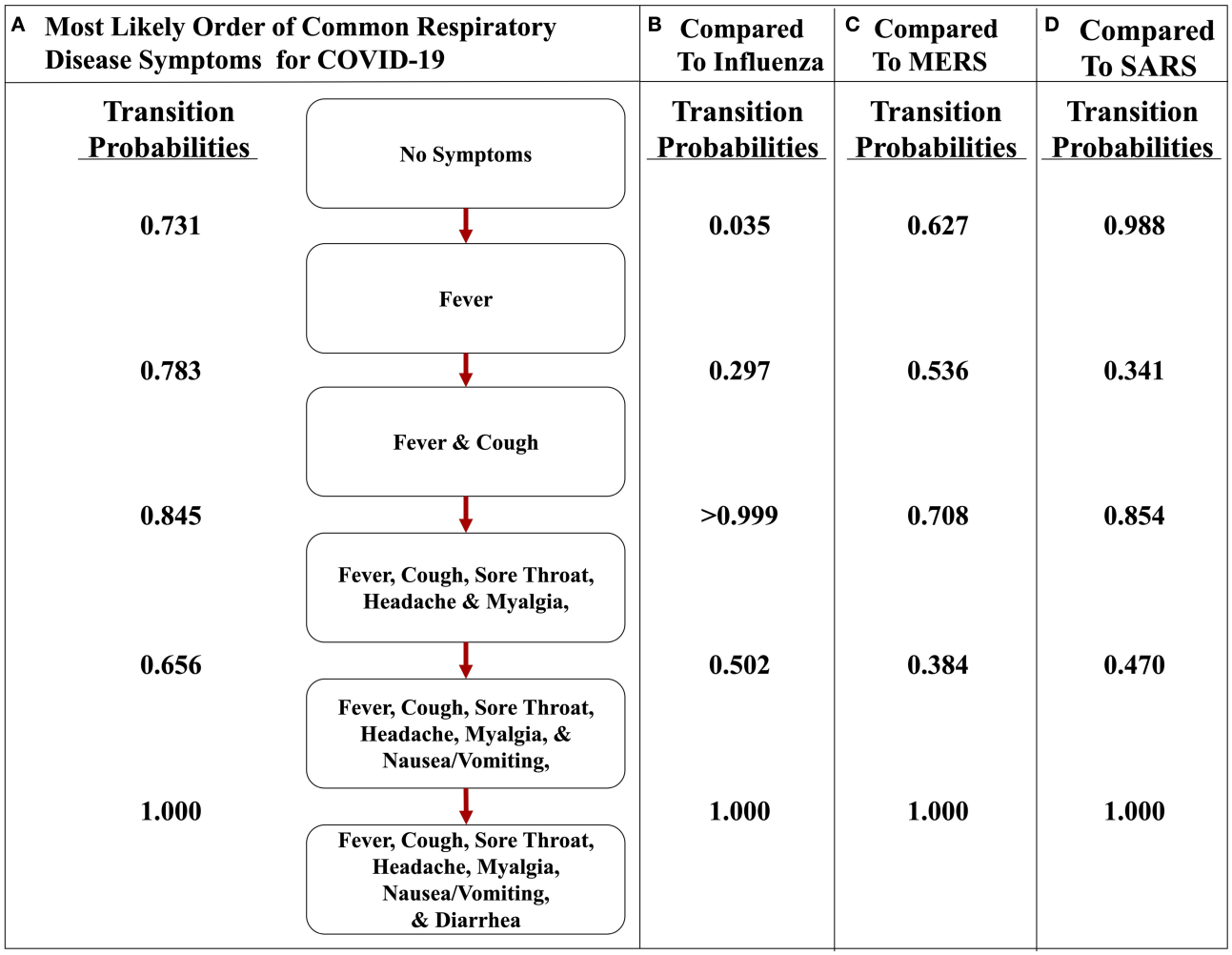


FIGURE 6 | The most likely path of symptoms in COVID-19 vs. influenza, MERS, and SARS. **(A)** The most likely path of seven common symptoms of COVID-19 with the transition probabilities listed between nodes. **(B)** The transition probabilities of the path of influenza. **(C)** The transition probabilities of the path of MERS. **(D)** The transition probabilities of the path of SARS. The most likely path here is determined from the transition probabilities listed between nodes for COVID-19.

analyzing influenza as it traverses down the most likely path of COVID-19, the transition probabilities observed are 0.5 or less (**Figure 6B**). However, in that same path, the transition probability from fever and cough to fever, cough, sore throat, headache, and myalgia is >0.999. This value displays how unlikely nausea/vomiting and diarrhea are to be initial symptoms of influenza. Additionally, when observing the most likely path of COVID-19, the first two symptoms seem to have a strong probability of occurring in the order of fever and then cough, with a likelihood of 0.731 (**Figure 5A**). However, the likelihood of cough occurring first in COVID-19 is 0.229, which is a low probability (**Figure 5A**). This observation further supports the hypothesis of fever occurring first and cough occurring second.

Recall and Selectivity When Linking First Symptom and Disease

The COVID-19 and influenza implementations of the Stochastic Progression Model suggest that there is a high likelihood of fever and cough occurring first, respectively. We desired to find metrics quantifying the possible link between first symptom and these two diseases. So, we determined the recall and the selectivity when using the initial symptom as an indicator of COVID-19 or influenza, with all other possible diseases excluded in a theoretical patient population. First, we simulated patient datasets using reported data that were independent from all previous work that we integrated in our analyses above (**Supplemental Table 5**) (20). Two simulated patient datasets were created to analyze COVID-19 and influenza separately to portray the specific link of each disease with the corresponding initial symptom that we determined, fever and cough, respectively. The simulated data contained information about the patients' state of disease (COVID-19, influenza or not) and their first symptom experienced. Based on the information of the first symptom alone, we categorized the simulated patient data as infected with COVID-19 or not and influenza or not. The recall was calculated as the number of simulated patients that we correctly identified as having the

TABLE 1 | Recall and selectivity of linking fever as a first symptom of patients with COVID-19.

	COVID-19			
	Recall		Selectivity	
	Mean	Standard deviation	Mean	Standard deviation
10 Patients out of 200	0.980	0.063	0.661	0.030
20 Patients out of 400	0.990	0.021	0.665	0.030
30 Patients out of 600	0.977	0.035	0.668	0.017
40 Patients out of 800	0.973	0.018	0.665	0.020
50 Patients out of 1,000	0.966	0.031	0.665	0.016

The mean and standard deviation of the recall and the selectivity of various simulations at different sample sizes that were each performed 10 times.

TABLE 2 | Recall and selectivity of linking cough as a first symptom of patients with influenza.

	Influenza			
	Recall		Selectivity	
	Mean	Standard deviation	Mean	Standard deviation
10 Patients out of 200	0.810	0.110	0.369	0.031
20 Patients out of 400	0.820	0.067	0.364	0.030
30 Patients out of 600	0.777	0.061	0.364	0.015
40 Patients out of 800	0.765	0.092	0.367	0.023
50 Patients out of 1,000	0.804	0.051	0.362	0.014

The mean and standard deviation of the recall and the selectivity of various simulations at different sample sizes that were each performed 10 times.

disease over the number of simulated patients that truly had the disease (27). Selectivity was defined here as the number of simulated patients that we correctly identified as not having the disease over the number of simulated patients that truly did not have the disease (28). For both diseases, we performed this analysis for five simulated samples of different sizes, each containing 5% infected individuals. We repeated this process 10 times and calculated the average and standard deviation across each sample size for both COVID-19 and influenza (**Tables 1**, **2**).

The recall ranges from 0.966 to 0.990 with a standard deviation of 0.031 and 0.021, respectively when analyzing the link between COVID-19 and fever as a first symptom. The maximum standard deviation of any sample size is 0.063 for the mean of 0.980. On the other hand, the selectivity of fever as a first symptom of COVID-19 ranges from 0.661 to 0.668 with a standard deviation of 0.030 and 0.020, respectively, and 0.030 is the maximum standard deviation with corresponding means of 0.661 and 0.665 (**Table 1**). As for cough as a first symptom of influenza, the recall ranges from 0.765 to 0.820 with corresponding standard deviations 0.092 and 0.067. The highest standard deviation is 0.110 with a mean of 0.810, and the selectivity ranges from 0.362 to 0.369 with standard deviations of 0.014 and 0.031, respectively, and the maximum standard deviation is 0.031 (**Table 2**).

The recall in both cases is lower than the selectivity, and this observation indicates that this analysis categorizes patients as infected when they are not, but the high recall indicates that most infected patients did align with the first symptom that we predicted. In the future, we expect to confirm this analysis with data on first symptoms, as opposed to simulated data, but the purpose of this analysis was to display that further study of order of symptoms might lead to earlier recognition.

DISCUSSION

In this study, we found evidence that supports the notion that there is a most common order of discernible symptoms in COVID-19 that is also different from other prominent respiratory diseases. The most likely initial symptom is fever in the three diseases studied that are caused by coronaviruses (i.e., COVID-19, SARS, and MERS) and cough in influenza. The most likely order of the four easily discernible symptoms is identical in MERS and SARS, but the most likely path of COVID-19 has one key difference. The first two symptoms of COVID-19, SARS, and MERS are fever and cough. However, the upper GI tract (i.e., nausea/vomiting) seems to be affected before the lower GI tract (i.e., diarrhea) in COVID-19, which is the opposite from MERS and SARS. In all diseases, we found that fever and cough occur before nausea/vomiting and diarrhea. When observing the set of seven symptoms including three subjective ones (i.e., sore throat, headache, and myalgia), we found that the initial symptoms of the most likely path are the same as in the most likely path of the four discernible symptoms. Also, in both the four and seven symptoms implementations, the GI tract symptoms are last. A separate MERS dataset included the initial symptoms of patients on admission, which listed the symptoms from highest to lowest probability as fever, myalgia, cough, and diarrhea (18). This order is similar to the most likely path that we determined. A very small percent of patients experienced diarrhea as an initial symptom. This report suggests that diarrhea as an early symptom indicates a more aggressive disease, because each patient in this dataset that initially experienced diarrhea had pneumonia or respiratory failure eventually (**Supplemental Table 3**). We propose that these patients may be experiencing a more aggressive form of the disease and have accelerated through the most likely path, having already experienced diarrhea. These findings align with another dataset provided for SARS, which also contained the percentage of the various symptoms to be reported first (**Supplemental Table 4**). The highest reported symptom is fever, followed by cough or dyspnea, and then finally, a small percent of patients reported diarrhea (19). This order confirms the most likely paths that we have determined. The observation that diarrhea was very uncommon as a first symptom and had a non-zero probability of occurrence is consistent with our analysis. This aligns with our hypothesis that early occurrence of diarrhea

implies that those patients may have a much more aggressive form of the disease.

The simulation data used to approximate the state and transition probabilities in the Stochastic Progression Model relies on the assumption that symptoms included in the model are independent. Using the definition of independence, we observed the individual probabilities of fever and cough in a dataset from a case study of influenza, and we found that the product of the individual probabilities of fever and cough is almost equal to the probability of both occurring (21). Considering this outcome, we proceeded under the assumption of independence, which we will reevaluate when more symptom data becomes available. We simulated combinations of symptoms for 500,000 patients, which we chose because it was the lowest attempted number that empirically produced the theoretical expected outcome for random frequency symptoms: that all paths would be equally likely, up to 100ths of a decimal place. We then utilized these simulated patients to approximate the state probabilities and transition probabilities described above.

This study supports the idea that symptoms occur in a predictable order, but future work is needed to improve aspects of the Stochastic Progression Model and confirm the results found here. Our finding that COVID-19 first presents with a fever supports the recommended measures by the CDC which state that the public should take their temperature at home and when entering facilities as an early checking method (29). This application of the Stochastic Progression Model may be improved if there were objective ways to measure the more subjective symptoms (i.e., sore throat, headache, and myalgia). Also, improved error calculations of the transition probabilities would lead to more accurate results. Our current error calculation is conservative, because when more symptoms were added, we observed that the error compounded as we progressed further down the paths (**Supplemental Figures 2–13**). The conservative error estimate creates issues in discerning the difference in probabilities of symptoms. Specifically, in implementations of seven symptoms, the likelihoods are more difficult to ascertain due to subjective reporting and compounding error calculations. Datasets that contain the order of symptoms for each patient would lower the error. Additionally, these sorts of datasets would better the approximations of the transition probabilities and increase accuracy. This improvement could be achieved by physicians implementing the practice of recording the order of occurrence of symptoms. With this information, we may approximate the likelihood of a patient acquiring a symptom based on their current symptoms with patient data instead of simulations based on frequency. Applying objective criteria for symptoms, improving error calculations, and collecting the order of symptoms would not only allow us to improve our findings here, but also allow the Stochastic Progression Model to predict orders of a larger set of symptoms. The optimal form of the Stochastic Progression Model would be developed by determining state probabilities from observed true frequencies of patients' symptoms and determining transition probabilities from the patients' true order of symptoms. However, until this data is available, improved approximations, simulations and error calculations are needed.

Furthermore, when analyzing fever as the first symptom of COVID-19, a low selectivity indicates a high Type I error (i.e., rate of false positive), and a high recall indicates a low Type II error (i.e., rate of false negative). We found a moderate selectivity value and as a result, a moderate Type I error in this case. This Type I error is acceptable in our use of investigating fever as an initial symptom of COVID-19, because it suggests that more people get tested who are not infected, rather than less people get tested who are infected, as with Type II error (30). We are not proposing initial symptom as a diagnostic test, but instead as a possible sign to get tested. COVID-19 outbreaks in clusters, and these unusual clusters of disease are characteristic of a pandemic disease that must be addressed immediately with aggressive testing to curb transmission (31).

The importance of knowing first symptoms is rooted in the need to stop the spread of COVID-19, a disease that is two to three times more transmissible than influenza and results in outbreaks of clusters (3, 4). There is a heightened risk in COVID-19 being passed on, so faster testing and social distancing are important, especially when social distancing and quarantine measures are relaxed. Our results assert that fever is the most likely symptom to occur first in symptomatic adult patients with COVID-19. We hope that the hypotheses generated in this work are tested with prospective clinical data to confirm that a cough occurs first more often in influenza and likewise fever in COVID-19. We believe that early detectors that any individual can recognize to seek medical attention earlier is useful. In addition, datasets that contain information of symptom order and strains of COVID-19 allow for further studies that may determine whether onset of symptoms vary in specific strains (32), and whether risk factors, such as obesity (33), and environmental factors, such as temperature (34) affect symptom order. To slow the spread of COVID-19, our results support the practice that fever should be tested before allowing entry to facilities and that those with fever should immediately seek medical attention for diagnosis and contact tracing. Such measures as these may help to reduce transmission despite the high contagion of SARS-CoV-2.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed for this study. These can be found here: https://www.who.int/publications-detail/report-of-the-who-china-joint-mission-on-coronavirus-disease-2019-(covid-19), https://www.nejm.org/doi/full/10.1056/NEJMoa2002032, https://jamanetwork.com/journals/jamainternalmedicine/fullarticle/485554, https://onlinelibrary.wiley.com/doi/full/10.1111/resp.13196, https://www.journalofinfection.com/article/S0163-44531630209-2/abstract, https://jamanetwork.com/journals/jama/fullarticle/196681, https://jamanetwork.com/journals/jama/fullarticle/2761044, https://www.cambridge.org/core/journals/infection-control-and-hospital-epidemio

logy/article/symptoms-of-influenza-virus-infection-in-hospitalized-patients/8F1B478BA4B861D356393EA77AD8B83B#.

AUTHOR CONTRIBUTIONS

JL and JH conceived the model. JL and JM conceived the project. JL created the model. JL, MM, and JM analyzed results. JL and MM wrote the manuscript. PK and JH supervised the project. All authors read, edited, and approved the final manuscript.

FUNDING

This work was supported by the National Cancer Institute (Award Number U54CA143906 and P30CA014089) and the Carol Vassiliadis fellowship. JL was supported by the USC Dana and David Dornsife College of Letters, Arts and Sciences, and the Schlegel Family Endowment Fellowship.

ACKNOWLEDGMENTS

We wish to thank Dr. Jorge Nieva for discussions and advisement and Libere Ndacayisaba for critical reading of the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2020.00473/full#supplementary-material

REFERENCES

1. Dong E, Du H, Gardner L. An interactive web-based dashboard to track COVID-19 in real time. *Lancet Infectious Dis.* (2020) 20:533–4. doi: 10.1016/S1473-3099(20)30120-1
2. Cascella M, Rajnik M, Cuomo A, Dulebohn SC, Di Napoli R. *Features, Evaluation and Treatment Coronavirus (COVID-19).* StatPearls Publishing (2020).
3. Anderson RM, Heesterbeek H, Klinkenberg D, Hollingsworth TD. How will country-based mitigation measures influence the course of the COVID-19 epidemic? *Lancet.* (2020) 395:931–4. doi: 10.1016/S0140-6736(20)30567-5
4. Sun K, Chen J, Viboud C. Early epidemiological analysis of the coronavirus disease 2019 outbreak based on crowdsourced data: a population-level observational study. *Lancet Digital Health.* (2020) 2:E201–8. doi: 10.1016/S2589-7500(20)30026-1
5. Sohrabi C, Alsafi Z, O'Neill N, Khan M, Kerwan A, Al-Jabir A, et al. World Health Organization declares global emergency: a review of the 2019 novel coronavirus (COVID-19). *Int J Surg.* (2020) 76:71–6. doi: 10.1016/j.ijsu.2020.02.034
6. Monto AS, Gravenstein S, Elliott M, Colopy M, Schweinle J. Clinical signs and symptoms predicting influenza infection. *Archiv Internal Med.* (2000) 160:3243–7. doi: 10.1001/archinte.160.21.3243
7. Yin Y, Wunderink RG. MERS, SARS and other coronaviruses as causes of pneumonia. *Respirology.* (2018) 23:130–7. doi: 10.1111/resp.13196
8. Organization WH, Organization WH. *Report of the WHO-China Joint Mission on Coronavirus Disease 2019 (Covid-19).* (2020). Available online at: https://www.who.int/docs/default-source/coronaviruse/who-china-joint-mission-on-covid-19-final-report.pdf (accessed April 12, 2020).
9. Reed C, Kim IK, Singleton JA, Chaves SS, Flannery B, Finelli L, et al. Estimated influenza illnesses and hospitalizations averted by vaccination—United States, 2013–14 influenza season. *MMWR Morbidity mortality Weekly Report.* (2014) 63:1151–4.
10. Newton PK, Mason J, Bethel K, Bazhenova LA, Nieva J, Kuhn P. A stochastic Markov chain model to describe lung cancer growth and metastasis. *PLoS ONE.* (2012) 7:34367. doi: 10.1371/journal.pone.0034637
11. Newton PK, Mason J, Bethel K, Bazhenova L, Nieva J, Norton L, et al. Spreaders and sponges define metastasis in lung cancer: a Markov chain Monte Carlo mathematical model. *Cancer Res.* (2013) 73:2760–9. doi: 10.1158/0008-5472.CAN-12-4488
12. Bazhenova L, Newton P, Mason J, Bethel K, Nieva J, Kuhn P. Adrenal metastases in lung cancer: clinical implications of a mathematical model. *J Thoracic Oncol.* (2014) 9:442–6. doi: 10.1097/JTO.0000000000000133
13. Newton PK, Mason J, Hurt B, Bethel K, Bazhenova L, Nieva J, et al. Entropy, complexity, and Markov diagrams for random walk cancer models. *Sci Rep.* (2014) 4:7558. doi: 10.1038/srep07558
14. Newton PK, Mason J, Venkatappa N, Jochelson MS, Hurt B, Nieva J, et al. Spatiotemporal progression of metastatic breast cancer: a Markov chain model highlighting the role of early metastatic sites. *NPJ Breast Cancer.* (2015) 1:1–9. doi: 10.1038/npjbcancer.2015.18
15. Eriksson K, Jonsson M, Sjöstrand J. Markov chains on graded posets. *Order.* (2018) 35:93–109. doi: 10.1007/s11083-016-9420-1
16. Guan WJ, Ni ZY, Hu Y, Liang WH, Ou CQ, He JX, et al. Clinical characteristics of coronavirus disease 2019 in China. *N Engl J Med.* (2020) 382:1708–20. doi: 10.1056/NEJMoa2002032
17. Chan MC, Lee N, Chan PK, To K, Wong RY, Ho W-S, et al. Seasonal influenza A virus in feces of hospitalized adults. *Emerg Infectious Dis.* (2011) 17:2038. doi: 10.3201/eid1711.110205
18. Ko J-H, Park GE, Lee JY, Lee JY, Cho SY, Ha YE, et al. Predictive factors for pneumonia development and progression to respiratory failure in MERS-CoV infected patients. *J Infection.* (2016) 73:468–75. doi: 10.1016/j.jinf.2016.08.005
19. Booth CM, Matukas LM, Tomlinson GA, Rachlis AR, Rose DB, Dwosh HA, et al. Clinical features and short-term outcomes of 144 patients with SARS in the greater Toronto area. *JAMA.* (2003) 289:2801–9. doi: 10.1001/jama.289.21.JOC30885
20. Wang D, Hu B, Hu C, Zhu F, Liu X, Zhang J, et al. Clinical characteristics of 138 hospitalized patients with 2019 novel coronavirus–infected pneumonia in Wuhan, China. *JAMA.* (2020) 323:1061–9. doi: 10.1001/jama.2020.1585
21. Van den Dool C, Hak E, Wallinga J, Van Loon A, Lammers J, Bonten M. Symptoms of influenza virus infection in hospitalized patients. *Infection Control Hospital Epidemiol.* (2008) 29:314–9. doi: 10.1086/529211
22. Ciomek K. *hasseDiagram: Drawing Hasse Diagram.* (2017). Available online at: https://CRAN.R-project.org/package=hasseDiagram (accessed April 09, 2020).
23. Team RC. *R: A Language and Environment for Statistical Computing.* R Foundation for Statistical Computing. (2018). Available online at: https://www.R-project.org/ (accessed February 13, 2019).
24. Varga Z, Flammer AJ, Steiger P, Haberecker M, Andermatt R, Zinkernagel AS, et al. Endothelial cell infection and endotheliitis in COVID-19. *Lancet.* (2020) 395:1417–8. doi: 10.1016/S0140-6736(20)30937-5
25. Wu Y, Xu X, Chen Z, Duan J, Hashimoto K, Yang L, et al. Nervous system involvement after infection with COVID-19 and other coronaviruses. *Brain Behavior Immunity.* (2020) 87:18–22. doi: 10.1016/j.bbi.2020.03.031
26. Diaz-Guimaraens B, Dominguez-Santas M, Suarez-Valle A, Pindado-Ortega C, Selda-Enriquez G, Bea-Ardebol S, et al. Petechial skin rash associated with severe acute respiratory syndrome coronavirus 2 infection. *JAMA Dermatol.* (2020) 156:820–2. doi: 10.1001/jamadermatol.2020.1741
27. Powers DMW. Evaluation: from precision, recall and fmeasure to ROC, informedness, markedness and correlation. *J Mach Learn Technol.* 2:37–63.
28. Lozano J, Santos J, Horrillo M. Classification of white wine aromas with an electronic nose. *Talanta.* (2005) 67:610–6. doi: 10.1016/j.talanta.2005.03.015
29. Ng Y, Li Z, Chua YX, Chaw WL, Zhao Z, Er B, et al. Evaluation of the effectiveness of surveillance and containment measures for the first 100 patients with COVID-19 in Singapore–January 2–February 29,

2020. *Centers Dis Control Prev*. (2020) 69:307–11. doi: 10.15585/mmwr.mm6911e1

30. Pericchi L, Pereira C. Adaptative significance levels using optimal decision rules: balancing by weighting the error probabilities. *Brazil J Probabil Statist.* (2016) 30:70–90. doi: 10.1214/14-BJPS257
31. Hitchcock P, Chamberlain A, Van Wagoner M, Inglesby TV, O'Toole T. Challenges to global surveillance and response to infectious disease outbreaks of international importance. *Biosecurity Bioterrorism.* (2007) 5:206–27. doi: 10.1089/bsp.2007.0041
32. Tang X, Wu C, Li X, Song Y, Yao X, Wu X, et al. On the origin and continuing evolution of SARS-CoV-2. *Natl Sci Rev.* (2020) 7:nwaa036. doi: 10.1093/nsr/nwaa036
33. Lighter J, Phillips M, Hochman S, Sterling S, Johnson D, Francois F, et al. Obesity in patients younger than 60 years is a risk factor for Covid-19 hospital admission. *Clin Infect Dis.* (2020) 71:ciaa415. doi: 10.1093/cid/ciaa415
34. Demongeot J, Flet-Berliac Y, Seligmann H. Temperature decreases spread parameters of the new covid-19 case dynamics. *Biology.* (2020) 9:94. doi: 10.3390/biology9050094

Conflict of Interest: MM is employed by the company Nexus Development PA LLC. JM is employed by the company NanoCarrier Co., Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Medicine

ORIGINAL RESEARCH
published: 13 August 2020
doi: 10.3389/fmed.2020.00490

A Simple Bayesian Method for Evaluating Whether Data From Patients With Rheumatic Diseases Who Have Been Under Chronic Hydroxychloroquine Medication Since Before the COVID-19 Outbreak Can Speak to Hydroxychloroquine's Prophylactic Effect Against Infection With SARS-CoV-2

*Serban C. Musca**

Université Rennes 2, Campus Villejean, Place du Recteur Henri Le Moal, CS 24307, Rennes Cedex, France

OPEN ACCESS

Edited by:
Abdallah Samy,
University of Kansas, United States

Reviewed by:
Aruni Wilson,
Loma Linda University, United States
Tatjana Pustahija,
Institute of Public Health of Vojvodina, Serbia

***Correspondence:**
Serban C. Musca
serban-claudiu.musca@univ-rennes2.fr

Specialty section:
This article was submitted to Infectious Diseases – Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: *27 April 2020*
Accepted: *20 July 2020*
Published: *13 August 2020*

Citation:
Musca SC (2020) A Simple Bayesian Method for Evaluating Whether Data From Patients With Rheumatic Diseases Who Have Been Under Chronic Hydroxychloroquine Medication Since Before the COVID-19 Outbreak Can Speak to Hydroxychloroquine's Prophylactic Effect Against Infection With SARS-CoV-2. Front. Med. 7:490. doi: 10.3389/fmed.2020.00490

No vaccine against infection by SARS-CoV-2 yet exists. Treatment by hydroxychloroquine (HCQ) medication, among others, has been proposed. However, prophylactic HCQ medication has been little evaluated. We propose to use data from patients with rheumatic diseases (RA, SLR) who have been chronically taking HCQ medication since before the COVID-19 outbreak (hereafter: HCQpa), in order to evaluate the potential of HCQ for preventing infection with SARS-CoV-2. This can be achieved with relative ease by considering whether COVID-19 prevalence is significantly lower in HCQpa than in the general population (i.e., all people that are not HCQpa). Even if COVID-19 prevalence is truly significantly lower in HCQpa, some HCQpa may still present with COVID-19 (lower prevalence does not mean a prevalence of zero). However, given a value for COVID-19 prevalence in the general population and a number of available HCQpa, one may compute the maximum number of HCQpa for that total number of HCQpa considered that can have COVID-19 in order to still be able to conclude a lower COVID-19 prevalence in HCQpa (i.e., if there is one more case of COVID-19 than that maximum number, the COVID-19 prevalence in the HCQpa cannot be said to be lower than in the general population). Because the COVID-19 prevalence in the general population is not known with precision, we will consider different general population prevalence values. Among these contemplated prevalence values, one is derived from the official total number of confirmed cases, others by computing the total number of cases from the number of fatal COVID-19 cases so far and considering different case fatality rates per total cases. Our analyses show that systematic testing

for COVID-19 in as few as 5,000 HCQpa is all that is needed for a test of whether HCQ has a prophylactic action against COVID-19, even for a COVID-19 prevalence value as low as 250 per 100,000, provided that test sensitivity is at least equal to its specificity. For higher COVID-19 prevalence values, the number of HCQpa needed is even lower.

Keywords: SARS-CoV-2 / COVID-19, hydroxychloroquine, chronic medication, prophylactic effect, systemic lupus erythematosus, rheumatoid arthritis, clemastine, cloperastine

INTRODUCTION

We all know that "we do not have antivirals, vaccines, antibody-based therapeutics, or specific treatments" (1) with which to avoid infection with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and to treat against coronavirus disease 2019 (COVID-19). Hydroxychloroquine (HCQ) medication has some effectiveness against COVID-19 *in vivo* (2–4) during the early symptomatic phase (3) and also in the long run, possibly because it "contribute[s] to attenuating the inflammatory response." (5) HCQ is not a new medicine, and clearly defined drug safety management recommendations for it exist (6). Given the global situation and the ongoing debate over whether HCQ medication is effective as a prophylactic means against SARS-CoV-2 and/or as a cure for COVID-19, we deemed worth exploring the feasibility of testing such a hypothesis. The questions that we ask here are as follows. Is there a large enough number of identifiable people who have been chronically taking HCQ medication since before the outbreak of SARS-CoV-2 as a treatment for other diseases? Is the number of those people large enough to allow for sound statistical inference? What results found in them would be suggestive of a prophylactic effect of HCQ against COVID-19?

A considerable number of people have indeed been chronically taking HCQ medication as a treatment for other diseases since before the outbreak of SARS-CoV-2. Thus, one could derive crucial information on the prophylactic effect of HCQ against infection with SARS-CoV-2 by analyzing data from patients chronically treated with HCQ since before the COVID-19 outbreak (hereafter: HCQpa). Indeed, HCQ is the treatment of choice for systemic lupus erythematosus (7) (SLE) and is also used as a drug in the management of rheumatoid arthritis (8) (RA). SLE prevalence is variable but is as high as 0.241% in the USA (9). RA prevalence is 0.24% globally (10) but is 0.5–1% in Europe and the USA (11, 12). The total number of HCQpa in a country with a population of millions thus constitutes a large, statistically interesting sample.

It may, of course, be that SLE, and RA patients are intrinsically more prone to infection in general and, in particular, with SARS-CoV-2, than all comers. However, finding that HCQpa are *less* prone to infection with SARS-CoV-2 than all comers who do not take HCQ medication would tend to prove that HCQ helps avoid infection with SARS-CoV-2.

METHODS

If HCQ has no prophylactic effect against infection with SARS-CoV-2, COVID-19 prevalence in HCQpa will not be statistically different from that in the general population (all comers who do not take HCQ medication; hereafter: pop_{gen}).

Inferential statistics allow an informed decision to be made based on data and allow a statement (e.g., "medicine X is effective against disease Y") to be made with a given degree of confidence. That degree of confidence is expressed as a probability and is usually 0.95. This probability of 0.95 means that when drawing a conclusion based on data, one has 95% chances of being correct and 5% chances of an erroneous (although suggested by the data) conclusion. If one wants to lower the chances of an erroneous conclusion, one can opt for a higher probability, for instance, 0.99. This is the value we opted for here.

If one wants to speak of the exact COVID-19 prevalence in HCQpa, one would have to test all HCQpa for SARS-CoV-2 infection in order to come up with an exact prevalence figure. Alternatively, one will have to test only a given number of HCQpa and express the result not as a value, but of an interval of values, because of the probabilistic nature of statistical inference. We decided to contemplate the more practicable second option and adopted the Bayesian credibility interval (13) as the interval of values used to draw our conclusions. The 0.99 credibility interval is an interval such that there is a 99% chance that the true value of the parameter under examination (here, COVID-19 prevalence in HCQpa) falls within its upper and lower bounds.

HCQ having a prophylactic effect against SARS-CoV-2 infection would manifest itself by a COVID-19 prevalence in HCQpa that is *lower* than the COVID-19 prevalence in the general population. Accordingly, we are interested in an *upper* bound of the COVID-19 prevalence in HCQpa 0.99 credibility interval that has a value that is still significantly *lower* than the COVID-19 prevalence in the general population.

As prevalence is "the proportion of cases of a specified condition that are fatal within a specified time" (14), that is

$$Prevalence = \frac{TNC}{PS}$$

with TNC being the total number of COVID-19 cases so far and PS the population size, one can reason in terms of TNC instead of prevalence (since PS is a constant). That is, for any given number of HCQpa, one can search for the maximum HCQpa TNC number that gives HCQpa COVID-19 prevalence a value that is still under the lower bound of a 0.99 credibility interval build based on the pop_{gen} COVID-19 prevalence value. If that TNC number is not reached (i.e., there are fewer COVID-19 cases for the total number of HCQpa considered), then one can conclude with a $< 1\%$ chance of error that HCQ has a prophylactic effect against infection with SARS-CoV-2. The upper bound of

the HCQpa TNC 0.99 credibility interval built on the pop_{gen} COVID-19 prevalence for a number N of HCQpa is given by finding the maximum value of TNC in HCQpa such that

$$1 - pbeta(pop_{gen}\text{COVID-19 prevalence value},\ \text{TNC in HCQpa},\ \text{N} - \text{TNC in HCQpa}) < 0.005$$

with *pbeta* being the cumulative probability function of a beta distribution (15, 16).

In the absence of systematic detection of COVID-19 in all of the individuals in a population, TNC is underestimated. Thus, computing the prevalence based on the reported TNC will result in a (vastly) underestimated prevalence value. To take that into account, we considered different TNC values. Because TNC itself is not meaningful, we considered different fatality rates per total cases, "the proportion of cases of a specified condition that are fatal within a specified time" (14) (hereafter: CFRTC), as the number of fatal COVID-19 cases so far (hereafter: NFCT) is known (14), and we know that

$$TNC = \frac{NFCT}{CFRTC}$$

RESULTS

In order to make this data simulation more concrete and personalized, we chose as an example country France, a European country that is affected by COVID-19 and has a population of around 65 million. As to the HCQpa that one would have to test if a study such as this one were actually run, it should ideally come from stratified sampling among the HCQpa. The data simulation results are based on the following data: NFCT = 22,890 and $TNC_{reported}$ = 161,665 (17); PS_{France} = 65,241,000 (18). Considering a conservative sum of SLE and RA prevalence of 0.6% (the exact figure may be higher, perhaps more than double that which we consider here, but we only intend to find out whether the prevalence sum translated in terms of the total number of cases yields a number large enough for inference purposes, so we consider the more conservative figure of 0.6%) yields about 400,000 SLE and RA patients in France. Supposing one in five of them has been medicated with HCQ entails a number of HCQpa of about 80,000. We also consider other, lower values for the number of HCQpa in order to test for the limits of the method.

Tables 1, **2** show the results for the case where the SARS-CoV-2 detection test has the same sensitivity and specificity value. The more general case (i.e., different sensitivity and specificity values) is discussed afterward.

With $TNC_{reported}$ = 161,665, pop_{gen} prevalence is about 248 per 100,000 (see first line of **Table 1**). For a number of HCQpa of 80,000, finding up to (and including) 163 HCQpa with COVID-19 disease would lead one to rightfully conclude that COVID-19 prevalence in HCQpa is lower than in pop_{gen}. Actually, the modal (the mean has no specific meaning attached to it and is thus uninterpretable for a beta distribution) COVID-19 prevalence in HCQpa in that case is of 202.205 per 100,000, as one can read in the corresponding cell in **Table 2**.

At the same pop_{gen} prevalence (i.e., same row in **Table 1**; here, first row, i.e., pop_{gen} prevalence of about 248 per 100,000), as the number of available HCQpa decreases from 80,000 to 50,000, to 20,000, etc., the maximum number of HCQpa that can be found with COVID-19 disease in order to still have a COVID-19 prevalence lower in the HCQpa than in pop_{gen} decreases, unsurprisingly. With only 2,000 HCQpa available, there is no way to evidence such a result, since even finding no HCQpa with COVID-19 disease does not warrant the inference that there is a lower COVID-19 prevalence in HCQpa than in pop_{gen}. Inspection of **Table 2** shows how the modal COVID-19 prevalence—computed for the maximum number of HCQpa that can be found with COVID-19 disease in order to still have a COVID-19 prevalence lower in the HCQpa than in pop_{gen}—decreases as the number of available HCQpa decreases.

We will now consider different pop_{gen} prevalence values, that is, look always in the column of a given number of available HCQpa and consider how results change as one looks at different rows (different prevalence values). The reason to consider different pop_{gen} prevalence values is that pop_{gen} prevalence computation depends on TMC, and TMC is likely greatly underestimated in the absence of systematic testing because it is the ratio of NFCT (likely accurate) to CFRTC (likely greatly overestimated). Hypothesizing a different, lower CFRTC (while holding NFCT constant at 22,890) has the consequence of *increasing* pop_{gen} COVID-19 prevalence. For instance, supposing CFRTC = 7% puts TNC value at 327,000 cases. In turn, this affects pop_{gen} COVID-19 prevalence, now at about 501 cases per 100,000. We also consider other lower CFRTC values that correspond to reported values for other countries (i.e., 5 and 2.5%), which yield pop_{gen} COVID-19 prevalence values of about 700 and 1,400 per 100,000, respectively. A Belgian study (19) using stratified sampling, published on March 26, 2020, found that 3–6% of adult people had antibodies against COVID-19, so we also considered lower CFRTC values that yield higher pop_{gen} COVID-19 prevalence values in the range between 2,000 and 6,000 per 100,000.

If we consider the case where 80,000 HCQpa are available, one can see from inspection of **Table 1** how the maximum number of HCQpa that can be found with COVID-19 disease in order to still have a COVID-19 prevalence lower in the HCQpa than in pop_{gen} increases dramatically with COVID-19 prevalence in pop_{gen}. For instance, if COVID-19 prevalence in pop_{gen} were indeed 6,000 per 100,000, one can have more than one HCQpa in 20 (4,628 out of 80,000) presenting with COVID-19 and still correctly conclude that COVID-19 prevalence is lower in HCQpa than in pop_{gen}—as one can see in **Table 2**, the modal COVID-19 prevalence in HCQpa would be then of about 5,784 per 100,000.

If we consider instead the case where only 2,000 HCQpa are available, the same is observed. Interestingly, however, one can see that for a COVID-19 prevalence in pop_{gen} as low as 500 per 100,000, it is possible now to evidence a COVID-19 prevalence that is lower than in pop_{gen}. Moreover, at the other extreme of pop_{gen} COVID-19 prevalence values considered, 6,000 per 100,000, one can have more than one HCQpa in 23 (93 out of 2,000) presenting with COVID-19 and still correctly conclude that COVID-19 prevalence is lower in the HCQpa than in

TABLE 1 | Maximum HCQpa TNC number such that one can conclude with < 1% chance of error that HCQ has a prophylactic effect against infection with SARS-CoV-2 ($MaxTNC_{.99}$), as a function of case fatality rate per total cases (CFRTC) and the number of available HCQpa.

CFRTC (%)	TNC	COVID-19 prevalence in the general population (per 100,000)		Number of available HCQpa					
				80,000	50,000	20,000	10,000	5,000	2,000
14.159	161,665	247.797		163	96	32	13	4	–
7	327,000	501.219		350	211	75	33	13	3
5	457,800	701.706		501	304	111	50	21	5
2.5	916,600	1,403.412	$MaxTNC_{.99}$	1,038	635	239	111	50	15
1.75426	1,304,824	2,000.006		1,499	920	350	165	75	25
1.16951	1,957,230	3,000.000		2,277	1,403	539	257	120	41
0.7017	3,262,078	5,000.043		3,842	2,375	921	445	211	76
0.58475	3,914,493	6,000.051		4,628	2,864	1,114	540	258	93

See text for details.

TABLE 2 | Modal value for COVID-19 prevalence per 100,000 in HCQpa for the critical values MaxTNC.99 given in **Table 1**, as a function of case fatality rate per total cases (CFRTC) and the number of available HCQpa.

CFRTC (%)	TNC	COVID-19 prevalence in the general population (per 100,000)		Number of available HCQpa					
				80,000	50,000	20,000	10,000	5,000	2,000
14.159	161,665	247.797		202.505	190.008	155.016	120.04	60.024	–
7	327,000	501.219		436.261	420.017	370.037	320.064	240.096	100.100
5	457,800	701.706		625.016	606.024	550.055	490.098	400.160	200.200
2.5	916,600	1,403.412	HCQpa modal COVID-19 prevalence	1,296.282	1,268.051	1,190.119	1,100.022	980.392	700.701
1.75426	1,304,824	2,000.006		1,872.547	1,838.074	1,745.175	1,640.328	1,480.592	1,201.201
1.16951	1,957,230	3,000.000		2,845.071	2,804.112	2,690.269	2,560.512	2,380.952	2,002.002
0.7017	3,262,078	5,000.043		4,801.370	4,748.190	4,600.460	4,440.888	4,201.681	3,753.754
0.58475	3,914,493	6,000.051		5,783.895	5,726.229	5,565.557	5,391.078	5,142.057	4,604.605

See text for details.

pop_{gen}—but now, as one can see in **Table 2**, the modal COVID-19 prevalence in HCQpa would be about 4,605 per 100,000.

Now let us consider what changes when the SARS-CoV-2 detection test has sensitivity and specificity values that differ one from another. This has to do with the false-positive rate (FPR) and false-negative rate (FNR), respectively, of the test. Indeed, the FPR of a test is the probability that the test gives a positive result in the absence of what it is used to detect. If we denote T a positive test result and notS the absence of SARS-CoV-2, the FPR of the test is P(T|notS). On the other hand, the specificity of a SARS-CoV-2 test is the probability that it gives a negative result (notT) in the absence of SARS-CoV-2, P(notT|notS). One can now see that the FPR and specificity sum up to one, P(T|notS) + P(notT|notS) = 1, so the further away the specificity of a test is from one, the higher its FPR, that is, the higher the probability of erroneously labeling a healthy person as infected with SARS-CoV-2. Similarly, the FNR of a test is the probability that the test will give a negative result when the person is in fact infected with SARS-CoV-2, P(notT|S). The sensitivity of that test, its probability of detecting SARS-CoV-2 when the person is infected with SARS-CoV-2, is P(T|S). One can now see that the FNR and sensitivity sum up to one, P(notT|S) + P(T|S) = 1, so the further away the sensitivity of a test is from one, the higher its FNR, that is, the higher the probability of erroneously labeling a person infected with SARS-CoV-2 as healthy.

What happens if FNR and FPR have the exact same (non-zero) value? Some healthy persons will be erroneously labeled as infected with SARS-CoV-2 because FPR is different from zero, and some of the persons infected with SARS-CoV-2 will be erroneously labeled as healthy because the FPR is different from zero, but over a large number of persons being tested, the number of persons with SARS-CoV-2 the test has missed and the number of healthy persons the test has labeled as infected with SARS-CoV-2 will be the same so that the total number of persons labeled as infected with SARS-CoV-2 will be correct. As $FNR = 1\text{- }sensitivity$ and $FPR = 1\text{- }specificity$, it is clear that $FNR = FPR$ when $sensitivity = specificity$.

In order to avoid the pitfall of concluding a prophylactic effect of HCQ, it is important that the FNR be at most equal to the FPR. Indeed, if the FNR were higher than the FPR, one would miss more persons infected with SARS-CoV-2 than the number of healthy persons that one would erroneously include as persons infected with SARS-CoV-2. One thus wants to have FNR ≤ FPR, that is, $1\text{- }sensitivity \leq 1\text{- }specificity$, which in turn requires $sensitivity \geq specificity$.

If *sensitivity* = *specificity*, one can use **Table 1** directly. Otherwise, a correction has to be made to the values of $MaxTNC_{.99}$ given in **Table 1** (and the COVID-19 prevalence in HCQpa from **Table 2** should not be used; instead, the correct COVID-19 prevalence in HCQpa would have to be recalculated using the corrected $MaxTNC_{.99}$ value). If *specificity* $\geq (\leq)$ *sensitivity*, then the correction entails subtracting (adding) *Number of available HCQpa* * *(specificity—sensitivity)*. For instance, for *sensitivity* = 0.995 and *specificity* = 0.9975, with 2,000 HCQpa and a COVID-19 prevalence of 1,403 per 100,000, the correction would entail subtracting 2,000 * (0.9975–0.995) = 5, so that $MaxTNC_{.99}$ would now have to be 10 (instead of 15; see **Table 1**) — also, the COVID-19 prevalence in HCQpa would then be 450.450 (instead of 700.701 from **Table 2**) per 100,000.

DISCUSSION

This study is concerned with the feasibility of drawing conclusions as to the prophylactic effect of HCQ against SARS-CoV-2 by taking into consideration data from people suffering from a disease that forced them to chronically take HCQ medication since before the outbreak of SARS-CoV-2. Firstly, we have established that there is a considerable number of people who are in this situation, as HCQ is used chronically in the treatment of SLE and RA. Indeed, for a country such as France (i.e., with a population of about 65 million), supposing a conservative prevalence sum for SLE and RA of 0.6% yields about 400,000 SLE and RA patients, so it is reasonable to suppose that some tens of thousands among them have been chronically taking HCQ medication since before the outbreak of SARS-CoV-2 (we will refer to those people as HCQpa, and we estimated their number to be of about 80,000).

We focused most of this study on the question of what results one should find in HCQpa in order to infer a prophylactic effect of HCQ against SARS-CoV-2. The obvious answer is that to begin with, one must find a COVID-19 prevalence in HCQpa lower than in the general population (defined as the population of all people who do not take HCQ medication). We then considered a Bayesian inference method that gives the maximum number of HCQpa—among a given number of available HCQpa—that could have the COVID-19 disease such that COVID-19 prevalence in HCQpa is still found to be significantly lower than in the general population. Because there is no consensus as to the value of COVID-19 prevalence in the general population, we considered different such values.

The interesting question that then arises is whether the number of available HCQpa is large enough to allow a lower COVID-19 prevalence in HCQpa than in the general population to be evidenced for all, for some, or for none of the values of COVID-19 prevalence in the general population. Another important question, for obvious practical reasons, is the minimum number of available HCQpa necessary in order to be able to reach such a conclusion if it were true. Our results (see **Tables 1**, **2**) show that the answer to the first question is affirmative and that the answer to the second is that quite few available HCQpa are needed.

This is a somewhat unsurprising result but is still interesting for its implication that, if COVID-19 prevalence is truly lower in HCQpa than in the general population, the higher the COVID-19 prevalence in the general population, the easier it is to evidence a lower COVID-19 prevalence in HCQpa than in the general population. With a SARS-CoV-2 detection test that has a sensitivity equal to its specificity, as few as 5,000 HCQpa would be enough to evidence a lower COVID-19 prevalence in HCQpa than in the general population (if that were true), even for a COVID-19 prevalence in the general population as low as 250 per 100,000. For a COVID-19 prevalence in the general population of 500 per 100,000 or higher, even a total of 2,000 HCQpa would be sufficient. **Table 1** gives, for each considered COVID-19 prevalence value in the general population and for each number of available HCQpa, a critical value, that is, the maximum number of HCQpa who can present with COVID-19 such that COVID-19 prevalence will still be lower in HCQpa than in the general population; **Table 2** displays the modal COVID-19 prevalence in HCQpa for those critical values.

We have also considered the case where the SARS-CoV-2 detection test has a sensitivity different from its specificity and, after analyzing the impact of this, we proposed a correction that, once applied, reduces this situation to that where the sensitivity of the test equals its specificity.

Finally, we consider the limits and potential extensions of the present study. A first limit, suggested by a reviewer, is that if the methodology we advocate here is put into practice and one does indeed find a lower COVID-19 prevalence in HCQpa than in the general population, there is no way to substantiate that the intake of HCQ by the HCQpa is the reason for the low prevalence of COVID-19. However, it seems to us difficult to argue that it is rather RA or SLR that instead protect against infection by SARS-CoV-2 (to our best knowledge, this has not been put forward in the literature). Still, the method outlined here applies to an observational type of study, and as such, even if the results show a lower COVID-19 prevalence in HCQpa than in the general population, one cannot conclude prophylactic effectiveness. To warrant such a conclusion, such findings ought to be confirmed in randomized clinical trials. To our best knowledge, however, the one published clinical trial (20) that aimed at testing for prophylactic effectiveness of HCQ and the others in progress or under review only tested *post*-exposure prophylaxis, that is, intake of HCQ following (i.e., subsequent to) exposure to SARS-CoV-2. The method we propose is to be used as a means to look for *pre*exposure prophylaxis; that is, it considers retrospective data inclusion in an observational study based on data from people who have been chronically taking HCQ medication since *before* the COVID-19 outbreak.

The methodology delineated here may be applied to other potential prophylactics or medication to test for their action against SARS-CoV-2. In that sense, the significance of the results presented here goes far beyond the question of whether HCQ may have a prophylactic effect and allows those who have been medicated with HCQ since before the COVID-19 outbreak to avoid infection with SARS-CoV-2. The only requirement is the existence of a large enough number of people who have been medicated with such potential drugs. Recently, after analyzing SARS-CoV-2 proteins and identifying which proteins from the human body they could interact with, a study (21) identified

many drugs already approved for use in humans that could target those interactions and thus help avoid infection with SARS-CoV-2 or fight COVID-19. On that list, there are quite mundane drugs, such as the antihistamines clemastine, and cloperastine, which have antiviral activity against SARS-CoV-2, and that many people with allergies take chronically. A first test of the action of these drugs against SARS-CoV-2 could be done much more rapidly with the methodology that we presented here than in a clinical trial—the R script that was used to derive the results is made available in the **Appendix**, which is to be found on the online article page. Also, the evaluation of the feasibility of such a test has already been done here. Indeed, everything that was concluded here as to the feasibility of evaluating HCQ as a prophylactic against SARS-CoV-2 can be said *mutatis mutandis* for another drug that a large enough number of people take chronically.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: John Hopkins Coronavirus Resource Center, https://coronavirus.jhu.edu/map.html, accessed April 26, 2020.

AUTHOR CONTRIBUTIONS

SM had the idea, did the analyses, and wrote the paper.

FUNDING

The funders had no role in study design, data analysis, data interpretation, or writing of the report. The author had full access to all data used in the study and had final responsibility for the decision to submit for publication.

ACKNOWLEDGMENTS

SM wishes to thank the reviewers for their consideration and their constructive remarks.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2020.00490/full#supplementary-material

REFERENCES

1. Poland GA. SARS-CoV-2: a time for clear and immediate action. *Lancet Infect Dis.* (2020) 20:531–2. doi: 10.1016/S1473-3099(20)30250-4
2. Colson P, Rolain JM, Lagier JC, Brouqui P, Raoult D. Chloroquine and hydroxychloroquine as available weapons to fight COVID-19. *Int J Antimicrob Agents.* (2020) 55:105932. doi: 10.1016/j.ijantimicag.2020.105932
3. Gautret P, Lagier JC, Parola P, Meddeb L, Mailhe M, Doudier B, et al. Hydroxychloroquine and azithromycin as a treatment of COVID-19: results of an open-label non-randomized clinical trial. *Int J Antimicrob Agents.* (2020) 56:105949. doi: 10.1016/j.ijantimicag.2020.105949
4. Zhou D, Dai SM, Tong Q. COVID-19: a recommendation to examine the effect of hydroxychloroquine in preventing infection and progression. *J Antimicrob Chemother.* (2020) 75:dkaa114. doi: 10.1093/jac/dkaa114
5. Liu J, Cao R, Xu M, Wang X, Zhang H, Hu H, et al. Hydroxychloroquine, a less toxic derivative of chloroquine, is effective in inhibiting SARS-CoV-2 infection *in vitro*. *Cell Discov.* (2020) 6:1–4. doi: 10.1038/s41421-020-0156-0
6. Fiehn C, Ness T, Weseloh C, Specker C, Hadjiski D, Detert J, et al. Safety management in treatment with antimalarials in rheumatology. Interdisciplinary recommendations on the basis of a systematic literature review. *Z Rheumatol.* (2020). doi: 10.1007/s00393-020-00785-4. [Epub ahead of print].
7. Goehner SK, Maurer B. New and established therapeutic options for the treatment of systemic lupus erythematosus. *Praxis.* (2020) 109:341–45. doi: 10.1024/1661-8157/a003443
8. Munster T, Gibbs JP, Shen D, Baethge BA, Botstein GR, Caldwell J, et al. Hydroxychloroquine concentration-response relationships in patients with rheumatoid arthritis. *Arthritis Rheum.* (2002) 46:1460–69. doi: 10.1002/art.10307
9. Rees F, Doherty M, Grainge MJ, Lanyon P, Zhang W. The worldwide incidence and prevalence of systemic lupus erythematosus: a systematic review of epidemiological studies. *Rheumatology.* (2017) 56:1945–61. doi: 10.1093/rheumatology/kex260
10. Cross M, Smith E, Hoy D, Carmona L, Wolfe F, Vos T, et al. The global burden of rheumatoid arthritis: estimates from the global burden of disease 2010 study. *Ann Rheum Dis.* (2014) 73:1316–22. doi: 10.1136/annrheumdis-2013-204627
11. Myasoedova E, Crowson CS, Kremers HM, Therneau TM, Gabriel SE. Is the incidence of rheumatoid arthritis rising?: results from Olmsted County, Minnesota, 1955-2007. *Arthritis Rheum.* (2010) 62:1576–82. doi: 10.1002/art.27425
12. Hunter TM, Boytsov NN, Zhang X, Schroeder K, Michaud K, Araujo AB. Prevalence of rheumatoid arthritis in the United States adult population in healthcare claims databases, 2004-2014. *Rheumatol Int.* (2017) 37:1551–57. doi: 10.1007/s00296-017-3726-1
13. Jaynes ET. Confidence Intervals vs Bayesian Intervals. In: Harper WL, Hooker CA, editors. *Foundations of Probability Theory, Statistical Inference, and Statistical Theories of Science,* Vol. I. Dordrecht: Reidel (1976). p. 175–257. doi: 10.1007/978-94-010-1436-6_6
14. Porta MA. *Dictionary of epidemiology*, 5th ed. Oxford: Oxford Univ. Press (2008).
15. Abramowitz M, Stegun IA. *Handbook of Mathematical Functions*. New York, NY: Dover Publ (1972).
16. Geffreys H. *Theory of Probability*, 3rd ed. Oxford: Oxford Univ. Press (1961).
17. John Hopkins Coronavirus Resource Center (2020). Available online at: https://coronavirus.jhu.edu/map.html (accessed April 26, 2020).
18. worldometer (2020). Available online at: https://www.worldometers.info/world-population/france-population/ (accessed April 7, 2020).
19. Sero-epidemiology COVID-19 in Belgium (2020). Retrieved from: https://www.uantwerpen.be/en/research-groups/vaxinfectio/corona-research/sero-epidemiology-co/ (accessed on May 24, 2020).
20. Boulware DR, Pullen MF, Bangdiwala AS, Pastick KA, Lofgren SM, Okafor EC, et al. A randomized trial of hydroxychloroquine as postexposure prophylaxis for covid-19. *N Engl J Med.* (2020). doi: 10.1056/NEJMoa2016638. [Epub ahead of print].
21. Gordon DE, Jang GM, Bouhaddou M, Xu J, Obernier K, White KM, et al. A SARS-CoV-2 protein interaction map reveals targets for drug repurposing. *Nature.* (2020) 583:459–68. doi: 10.1038/s41586-020-2286-9

Conflict of Interest: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

REVIEW
published: 13 August 2020
doi: 10.3389/fmicb.2020.01818

Antivirals Against Coronaviruses: Candidate Drugs for SARS-CoV-2 Treatment?

*Igor de Andrade Santos[1], Victória Riquena Grosche[1,2], Fernando Rodrigues Goulart Bergamini[3], Robinson Sabino-Silva[4] and Ana Carolina Gomes Jardim[1,2]**

[1] Laboratory of Virology, Institute of Biomedical Science, Federal University of Uberlândia, Uberlândia, Brazil, [2] Institute of Biosciences, Language and Exact Sciences, São Paulo State University, São José do Rio Preto, Brazil, [3] Laboratory of Synthesis of Bioinspired Molecules, Institute do Chemistry, Federal University of Uberlândia, Uberlândia, Brazil, [4] Department of Physiology, Institute of Biomedical Sciences, Federal University of Uberlândia, Uberlândia, Brazil

Coronaviruses (CoVs) are a group of viruses from the family *Coronaviridae* that can infect humans and animals, causing mild to severe diseases. The ongoing pandemic of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) represents a global threat, urging the development of new therapeutic strategies. Here we present a selection of relevant compounds that have been described from 2005 until now as having *in vitro* and/or *in vivo* antiviral activities against human and/or animal CoVs. We also present compounds that have reached clinical trials as well as further discussing the potentiality of other molecules for application in (re)emergent CoVs outbreaks. Finally, through rationalization of the data presented herein, we wish to encourage further research encompassing these compounds as potential SARS-CoV-2 drug candidates.

Keywords: antivirals, coronaviruses, COVID-19, SARS-CoV-2, treatment

OPEN ACCESS

Edited by:
Anna Kramvis, University of the Witwatersrand, Johannesburg, South Africa

Reviewed by:
Olivier Terrier, UMR 5308 Centre International de Recherche en Infectiologie (CIRI), France
Maryam Dadar, Razi Vaccine and Serum Research Institute, Iran

***Correspondence:**
Ana Carolina Gomes Jardim
jardim@ufu.br

Specialty section:
This article was submitted to Virology, a section of the journal Frontiers in Microbiology

Received: *21 April 2020*
Accepted: *10 July 2020*
Published: *13 August 2020*

Citation:
Santos IA, Grosche VR, Bergamini FRG, Sabino-Silva R and Jardim ACG (2020) Antivirals Against Coronaviruses: Candidate Drugs for SARS-CoV-2 Treatment? Front. Microbiol. 11:1818. doi: 10.3389/fmicb.2020.01818

INTRODUCTION

Coronaviruses (CoVs) were first identified in 1960 (Kahn and McIntosh, 2005) and were classified as members of the family *Coronaviridae*. CoVs are enveloped, single-stranded RNA viruses with a genome varying from 25 to 32 kb (Payne, 2017). The viral structure is primarily formed by the structural spike (S), membrane (M), envelope (E), and nucleocapsid (N) proteins. The S, M, and E proteins are embedded in the viral envelope, which is a lipid bilayer derived from the host cell membrane. The N protein, on the other hand, interacts with the viral RNA into the core of the virion (**Figure 1**; Fehr and Perlman, 2015).

These viruses can infect vertebrate animals, causing acute to chronic diseases in the respiratory, cardiac, enteric, and central nervous systems, both in animals and humans (Weiss and Navas-Martin, 2005). In animals, the most common CoVs are infectious bronchitis virus (IBV), feline CoV (FeCoV), and mouse hepatitis virus (MHV), which infect chickens, felines, and rodents, respectively (Cui et al., 2019). To date, there are seven known CoVs that cause diseases in humans: HCoV-229E, HCoV-NL63, HCoV-OC43, HCoV-HKU1, severe acute respiratory syndrome coronavirus (SARS-CoV), Middle East respiratory syndrome coronavirus (MERS-CoV) and, most recently, SARS-CoV-2 (Graham et al., 2013; CDC, 2020a). The CoVs HCoV-229E, HCoV-NL63, HCoV-OC43, and HCoV-HKU1 cause mild symptoms, similar to a common cold (Payne, 2017). However, SARS-CoV, MERS-CoV, and SARS-CoV-2 can cause mild to severe symptoms

related to upper respiratory infection such as fever, cough, dyspnea, pneumonia, and acute respiratory distress syndrome (ARDS), ultimately leading to death (Lai et al., 2020). The severe clinical condition generated especially by SARS-CoV-2 has been burdening public health systems worldwide (Hsu et al., 2020), evidencing the mandatory need for further research encompassing antiviral treatment against CoVs, which has somehow, until recently, been relatively ignored by broad pharmaceutical and medicinal fields (Lu et al., 2015; Cui et al., 2019).

CoVs are linked to a zoonotic transmission due to their ability to infect different species. This can lead to host jumps, allowing the emergence of new coronaviruses such as SARS-CoV, MERS-CoV, and SARS-CoV-2 (Lu et al., 2015; Reusken et al., 2016; Andersen et al., 2020). The transmission of CoVs is based on the fecal-oral route in animals (Kipar et al., 2010). In humans, CoV transmission occurs by direct contact with droplets when infected and recipient individuals are in close contact (about one meter). These infectious oral and respiratory droplets produced by talking, coughing, sneezing need to contact the mucosae (mouth and nose) or conjunctiva (eyes) of the recipient person. Additionally, indirect transmission can occur by touching a surface with viable CoV and subsequent contact with mouth, nose, or eyes (van Doremalen et al., 2020). Viral particles may remain viable on surfaces for several days, increasing the probability of infection by third parties (van Doremalen et al., 2020).

Recently, the emergence of SARS-CoV-2 was related to zoonotic transmission, but it is still not clear how this virus was first transmitted to humans (Andersen et al., 2020; Gorbalenya et al., 2020b). By phylogenetic analysis, the SARS-CoV-2 was grouped within bat SARS-related coronaviruses, suggesting that a host jump occurred (Cao et al., 2020a; Lai et al., 2020). Alarmingly, the high transmissibility of this new CoV allowed the rapid and efficient spread of the virus across the world so that it became a pandemic disease in just a few months (CDC, 2020a; Wu et al., 2020).

Due to the novelty of this disease, there is a lack of understanding of the SARS-CoV-2 replication process in host cells. The general mechanisms of entry into the host cell, replication, and release follow characteristics that have been described for other CoVs and have been partially confirmed for SARS-CoV-2. To date, it is known that the SARS-CoV-2 virion entries the host cells by the attachment of the S protein with angiotensin-converting enzyme 2 receptor (ACE2), defining SARS-CoV-2 tropism for cells that express this receptor, such as pulmonary, hepatic, gastrointestinal, and renal human cells (Chu et al., 2020; Hoffmann et al., 2020; Tai et al., 2020). The interaction of ACE2 with the receptor-binding domain (RBD) of the S protein triggers virion endocytosis and the formation of an endosome (Rabi et al., 2020). The S protein possesses two subunits, S1 and S2 (Walls et al., 2020). During endocytosis, an acid-dependent proteolytic cleavage of the S1 protein by cellular proteases, like cathepsin, TMPRRS2, and trypsin, exposes the S2 subunit, a fusion peptide that allows the fusion of the viral envelope with the endosome membrane, and consequently, releases the capsid into the cell cytoplasm (Belouzard et al., 2009; Matsuyama et al., 2020). In the cytoplasm, the CoV viral genome is uncoated, and the viral RNA is released. The positive-sense RNA viral genome is translated to produce nonstructural proteins (nsps) from two open reading frames (ORFs), ORF1a and ORF1b. The ORF1a encodes the polyprotein pp1a that is cleaved in 11 nsps, while the ORF1b encodes the polyprotein pp1ab, which is cleaved into 15 nsps. The proteolytic cleavage is performed by viral proteases nsp3 and nsp5 (Yogo et al., 1977; Lai and Stohlman, 1981; Kim et al., 2020). The nsps assemble to form a replicase-transcriptase complex (RTC) responsible for RNA synthesis, replication, and transcription of nine subgenomic RNAs (sgRNAs) (Fehr and Perlman, 2015; Chen W.-H. et al., 2020; Kim et al., 2020). The sgRNAs act as mRNAs for structural and accessory genes localized downstream of the replicase polyproteins. SARS-CoV-2 has six accessory proteins: 3a, 6, 7a, 7b, 8, and 10 (Kim et al., 2020). The structural proteins S, E, and M are translated from the sgRNAs and forwarded to the endoplasmic reticulum (ER) and are subsequently inserted into an intermediate compartment of ER with Golgi (ERGIC). There, viral genomes are encapsulated by N proteins and assembled with the structural proteins to form virions (Siu et al., 2008; Fehr and Perlman, 2015; Li et al., 2020). The M proteins bind to E protein and nucleocapsid, and then, the S protein is incorporated, forming a complete virion. Finally, the virions are transported to the cell surface in vesicles and released in a pathway mediated by exocytosis (**Figure 2**; Fehr and Perlman, 2015; Kim et al., 2020; Li et al., 2020).

It is important to emphasize that SARS-CoV-2 shows different epidemiological and clinical features from the epidemics of SARS-CoV and MERS-CoV (Ceccarelli et al., 2020; Gorbalenya et al., 2020a,b). The high transmissibility of SARS-CoV-2 may be related to its entry into host cells (Sun et al., 2020). Although both SARS-CoV and SARS-CoV-2 glycoprotein S attach to ACE2 to enter the host cells, the binding affinity of SARS-CoV-2 is higher, thus enhancing its infectivity (Sun et al., 2020; Yan et al., 2020). Despite the relative homology between S1 and S2 amino acid sequences, a 1.2 Å root-mean-square deviation at the 417 position (Lusvarghi and Bewley, 2016) of S2 protein in SARS-CoV-2 may be related to its higher infectiveness, contributing to a 10- to 20-fold higher kinetic affinity of SARS-CoV-2 ectodomain, as evidenced by Wrapp and co-workers, employing surface plasmon resonance measurements (Wrapp et al., 2020).

Considering the particularities of SARS-CoV-2 and the emergency caused by its outbreak, several strategies have been adopted to develop therapeutics and prophylactic measures against this virus. The strategies employed in these developments include: (i) utilization of bioinformatics for the prediction and investigation of potential ligands toward target molecules in the viral structure and/or replication (Ahmed et al., 2020, 2; Jeon et al., 2020, 2); (ii) employment of cell culture systems, permissive to CoVs (Caly et al., 2020; Liu et al., 2020), associated with pseudo particles, subgenomic replicons and/or full-length CoVs, seeking to assess cellular response or the effects of the compounds on the viral replicative cycle (Roberts et al., 2006; Hoffmann et al., 2020); (iii) the use of animal models, such as mice, mouse, guinea pig, hamster and non-human primates, for evaluating therapeutic options or antibody production in immunization (Natoli et al., 2020; Sheahan et al., 2020b), and (iv) clinical trials assessing

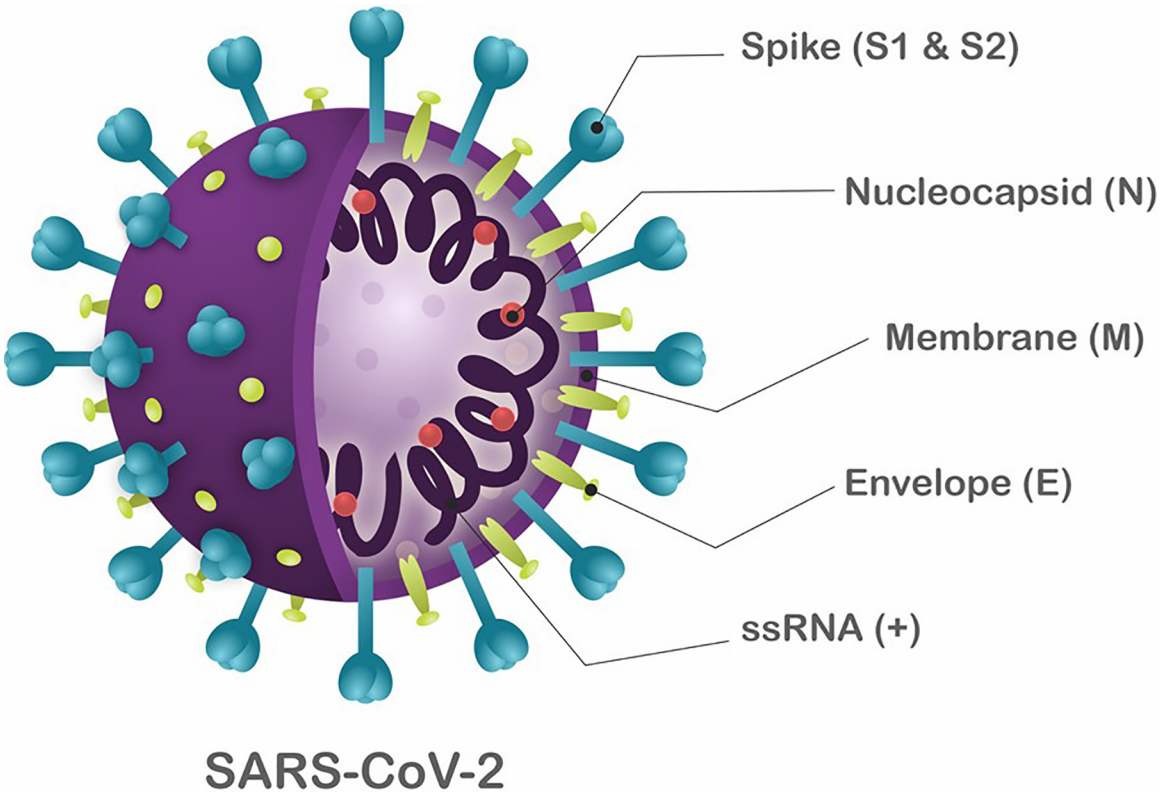


FIGURE 1 | Schematic structure of SARS-CoV-2. The viral structure is primarily formed by the structural proteins such as spike (S), membrane (M), envelope (E), and nucleocapsid (N) proteins. The S, M, and E proteins are all embedded in the viral envelope, a lipid bilayer derived from the host cell membrane. The N protein interacts with the viral RNA in to the core of the virion.

the administration, distribution, metabolism, and toxicity profiles (ADMeTox) of potential therapeutics as well as immunization effects in humans (Clark et al., 2019).

Based in previous results in vaccine development for MERS-CoV and SARS-CoV and the similarity of those viruses with SARS-CoV-2 (Dhama et al., 2020), the current vaccine candidates are more focused on the S protein, since is a major inducer of neutralizing antibodies in infected patients (Walls et al., 2020). For this reason, efforts are concentrated on using approaches such as mRNA, DNA, viral vectors, or virus-like particles vaccines with a full-length S protein or S1 receptor-binding domain (RBD) to stimulate immune response and immunization (Ahmed et al., 2020; Chen Y. et al., 2020). The most promising vaccines are: (i) adenovirus-vectored AZD1222 produced by Oxford University (Thomas, 2020), a vaccine that is currently in clinical phase 3, being tested in several countries, including the United States, Brazil, and countries in Asia and Africa; (ii) mRNA-1273 associated with a lipidic nanoparticle (NCT04283461), which is currently in clinical phase 2; and (iii) inactivated virus vaccine, which is currently in clinical phase 1 (Mullard, 2020; Tu et al., 2020).

The high transmissibility and viral variability of the novel SARS-CoV-2, along with the lack of a vaccine or drugs to treat the infected patients, threaten the global health system. In this context, the development of effective antivirals is critical to provide short-term therapies able to reduce the severity of clinical outcomes of coronavirus disease 2019 (COVID-19) and to reduce the spread of SARS-CoV-2. Here, we summarize compounds described, from 2005 to date, to possess antiviral activity *in vitro* and/or *in vivo* against CoVs and critically compare molecules that could be further investigated by their clinical applicability (**Table 1**). We also discuss the compounds that have reached clinical trials (**Table 2**) as well as the potentiality of other molecules for application in (re)emergent CoVs outbreaks. Finally, we aim to encourage further research encompassing these compounds as potential SARS-CoV-2 drug candidates.

INHIBITORS OF THE CoV REPLICATIVE CYCLE

Inhibitors of CoV Entry Into Host Cells

The entry of human CoVs into the host cells is mainly related to the binding of viral S protein to the ACE2 receptor (Prabakaran et al., 2004; Sun et al., 2020). Therefore, it is reasonable to hypothesize that compounds affecting this interaction could be potential antivirals (Prabakaran et al., 2004).

In this context, a survey encompassing *in silico* studies of more than 140 thousand potential S-protein-inhibiting drugs indicated that the molecule *N*-(2-aminoethyl)-1 aziridineethanamine

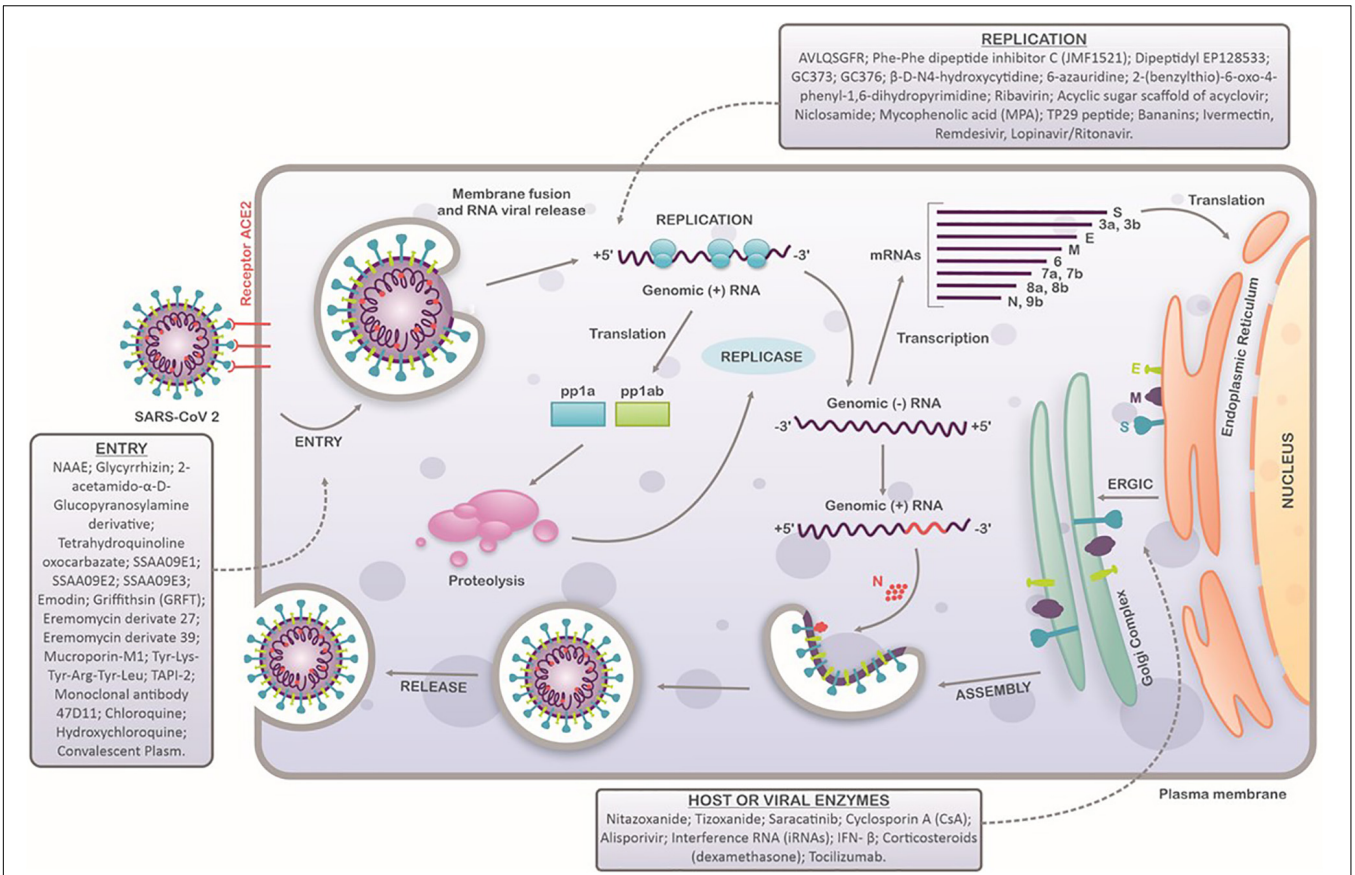


FIGURE 2 | Schematic representation of SARS-CoV-2 replication cycle in host cells. SARS-CoV-2 attaches to the host cells by interaction between the ACE2 receptors and spike proteins. After entry, viral uncoating process results in the release of viral genome and replication stage occurs (translation and transcription). Structural proteins are produced in intermediate compartment of endoplasmic reticulum with Golgi complex and forwarded to assembly, packaging and virus release. Compounds with antiviral activity against SARS-CoV-2 are indicated in each step of virus replication cycle.

(NAAE) showed the highest docking grade (-23.7 kcal/mol) (Huentelman et al., 2004b). The activity of NAAE was further confirmed by employing an *in vitro* enzymatic inhibitory assay, using a human recombinant ACE2. In this assay, ACE2 removed the C-terminal dinitrophenyl moiety that quenched the inherent fluorescence of the 7-methoxycoumain group, increasing the fluorescence when ACE2 was active (Huentelman et al., 2004b). The results showed that NAAE inhibited the ACE2 enzymatic activity with the half maximal inhibitory concentration (IC_{50}) of 57 μmol mL^{-1} (Huentelman et al., 2004b). In addition, 293T cells expressing ACE2 receptor were incubated with NAAE and then with S glycoprotein-expressing 293T cells, and measurement of β-galactosidase activity (reported gene in cell-cell fusion) was performed. NAAE at 0.5 μM inhibited 50% of SARS-CoVs spike protein-mediated cell fusion, suggesting that NAAE might be a candidate for treating SARS infection by impairing viral attachment via interference with ACE2 (Huentelman et al., 2004b). However, a detailed explanation of how NAAE is a more efficient ligand to ACE2 than other compounds was not attempted by the authors.

Ramos-Tovar and Muriel reported the antiviral activity of Glycyrrhizin (GL), a major constituent from licorice root (Ramos-Tovar and Muriel, 2019), which was able to inhibit SARS-CoV entry into Vero cells with an effective concentration of 50% (EC_{50}) of 300 mg L^{-1} and a cytotoxicity concentration of 50% (CC_{50}) of >20.000 mg L^{-1}. GL was less effective when the administration occurred during the viral adsorption period than when it was administered after entry into host cells. Cumulative effects were observed when this compound was administered both during and after entry into host cells, which indicates a significantly potent inhibitor against the virus under the tested conditions (Cinatl et al., 2003). Additionally, the antiviral activity of 15 GL derivates against SARS-CoV was assessed (Hoever et al., 2005). Conjugation on both acidic moieties of the GL disaccharide group with 2-acetamido-α-D-glucopyranosylamine, benzylcysteine, and Gly-Leu peptide generated compounds with an increase of 10- to 70-fold in anti-SARS-CoV activity when compared to GL itself (Hoever et al., 2005). For the case of 2-acetamido-α-D-glucopyranosylamine derivative, it was speculated that viral entry was inhibited through *N*-acetylglycosamine binding onto S-protein carbohydrates. Other derivatives such as the introduction of heterocyclic amides such as 6-amine-thiouracil induced a higher cytotoxicity profile.

TABLE 1 | Compounds with antiviral activity against human and animal coronaviruses.

Compound	Inhibition step	EC50 or inhibition (%)	CoVs	Advantges and/or limitations	References
NAAE	Entry	0.5 μM	SARS-CoV	Synthetic molecule, evaluated *in silico*, easily produced but lacks *in vivo* assays	Huentelman et al., 2004b
Glycyrrhizin	Entry	300 mg L^{-1}	SARS-CoV	Natural molecule, highly tolerated but lacks *in vivo* assays	Cinatl et al., 2003
2-acetamido-α-D-Glucopyranosylamine derivative	Entry	40 μM	SARS-CoV	Semi-synthetic molecule, highly tolerated, and more potent inhibitor but lacks *in vivo* assays	Hoever et al., 2005
Tetrahydroquinoline oxocarbazate (CID 23631927)	Entry (Cathepsin L)	273 nM	SARS-CoV	Synthetic molecule, highly tolerated, easily produced but lacks *in vivo* assays	Shah et al., 2010
SSAA09E1	Entry	6.7 μM	SARS-CoV	Synthetic molecule, highly tolerated, easily produced but lacks *in vivo* assays	Adedeji et al., 2013
SSAA09E2	Entry	3.1 μM	SARS-CoV	Synthetic molecule, highly tolerated, easily produced but lacks *in vivo* assays	Adedeji et al., 2013
SSAA09E3	Entry	9.7 μM	SARS-CoV	Synthetic molecule, highly tolerated, easily produced but lacks *in vivo* assays	Adedeji et al., 2013
Emodin	Entry and Post-Entry	50 μM	SARS-CoV	Natural molecule, highly tolerated but lacks *in vivo* assays	Ho et al., 2007; Schwarz et al., 2011
Griffithsin (GRFT)	Entry	0.16 μg mL^{-1}	HCoV-OC43	Natural molecule, highly tolerated, with a broad-spectrum effect (human and animal CoVs); protected against infection and improved survival in animal assay (Balb/c)	O'Keefe et al., 2010
	Entry	0.18 μg mL^{-1}	HCoV-229E		
	Entry	0.61 μg mL^{-1}	SARS-CoV		
	Entry	<0.032 μg mL^{-1}	HCoV-NL63		
	Entry	0.057 μg mL^{-1}	BCoV		
	Entry	0.23 μg mL^{-1}	MHV		
Eremomycin derivate 27	Entry	5.4 μM	FIPV	The precursor molecule (Eremomycin) is used to treat bacterial infections; may facilitate clinical assays, but knowledge of the mechanism of action is lacking	Balzarini et al., 2006
	Entry	14 μM	SARS-CoV		
Eremomycin derivate 39	Entry	12 μM	FIPV		
	Entry	22 μM	SARS-CoV		
Mucroporin-M1	Entry	14.46 μg mL^{-1}	SARS-CoV	Synthetic molecule, moderately tolerated, easily produced but lacks *in vivo* assays	Li et al., 2011
Tyr-Lys-Tyr-Arg-Tyr-Leu	Entry	14 mM	SARS-CoV	Synthetic molecule specifically designed to bind S protein of SARS-CoV; highly tolerated, does not impair ACE2 activity but lacks *in vivo* assays	Struck et al., 2012
	Entry	14 mM	HCoV-NL63		
TAPI-2	Entry	65%	SARS-CoV	Good effects in vitro assays but had no effect on *in vivo* assays	Haga et al., 2010
Monoclonal antibody 47D11	Entry	0.57 μg mL^{-1}	SARS-CoV-2	Human antibody, specifically to SARS-CoV-2, highly tolerated and easily applicable	Wang et al., 2020a
AVLQSGFR	Replication	2.7×10^{-2} mg mL^{-1}	SARS-CoV	Synthetic molecule, highly tolerated, easily produced but lacks *in vivo* assays	Gan et al., 2006
Phe-Phe dipeptide inhibitor C (JMF1521)	Replication	0.18 μM	SARS-CoV	Synthetic molecule, highly tolerated, easily produced but lacks *in vivo* assays	Shie et al., 2005
Dipeptidyl EP128533	Replication	3.6 μM or 1.4 μg mL^{-1}	SARS-CoV	Synthetic molecule, highly tolerated, easily produced but has contrasting effects in the literature and did not inhibit the virus in *in vivo* assays	Zhang et al., 2006; Day et al., 2009
GC373	Replication	0.2 μM	HCoV-229E	Synthetic molecule, highly tolerated, easily produced, seems to interact with SARS-CoV 3CLpro, but there are no *in vivo* assays	Kim et al., 2012, 2013
		0.3 μM	FIPV		
		2 μM	MHV		
		0.3 μM	TGEV		
		0.7 μM	BCV		
		0.15 μM	FCoV-WSU		

(Continued)

TABLE 1 | Continued

Compound	Inhibition step	EC50 or inhibition (%)	CoVs	Advantges and/or limitations	References
GC376	Replication	0.15 μM	HCoV-229E	Synthetic molecule, highly tolerated, easily produced, seems to interact with SARS-CoV 3CLpro, but there are no *in vivo* assays	Kim et al., 2012, 2013
		0.2 μM	FIPV		
		1.1 μM	MHV		
		0.15 μM	TGEV		
		0.6 μM	BCV		
		0.40 μM	FCoV-WSU		
6-azauridine	Replication	32 nM	HCoV-NL63	Synthetic molecule, highly tolerated, easily produced, but there are no *in vivo* assays	Pyrc et al., 2006
2-(benzylthio)-6-oxo-4-phenyl-1,6-dihydropyrimidine	Replication	NE	SARS-CoV	Synthetic molecule, highly tolerated, easily produced, but there are no in vivo assays	Ramajayam et al., 2010
β-D-N^4-hydroxycytidine	Replication	10 μM	SARS-CoV	Synthetic molecule, highly tolerated, easily produced, and improved pulmonary function and decreased viral load in lung of infected mice	Barnard et al., 2004; Sheahan et al., 2020b
	Replication	400 nM	HCoV-NL63		
	Replication	0.08–0.3 μM	SARS-CoV-2		
	Replication	0.024 μM	MERS-CoV		
Ribavirin	Replication	20 $\mu g\ mL^{-1}$	SARS-CoV	Synthetic molecule, highly tolerated, easily produced, good results in MERS-CoV. However, meta-analyses indicate limited efficacy.	Saijo et al., 2005; Barnard et al., 2006
Acyclic sugar scaffold of acyclovir	Replication	23 μM	MERS-CoV	Synthetic molecule, highly tolerated, easily produced, but there are no *in vivo* assays	Peters et al., 2015
		8.8 μM	HCoV-NL63	Synthetic molecule, highly tolerated, derivate from Acyclovir, easily produced, but there are no in vivo assays	
Niclosamide	Replication	0.1 μM	SARS-CoV	Drug already in use to treat helminthic infections; good inhibition *in vitro*	Wu et al., 2004; Wen et al., 2007
Mycophenolic acid (MPA)	Replication	2.87 μM	MERS-CoV	Good effects *in vitro* with MERS-CoV but did not inhibit SARS-CoV in *in vitro* and *in vivo* assays	Cinatl et al., 2003; Barnard et al., 2006; Hart et al., 2014
TP29 peptide	Replication	60 μM	MHV	Inhibited two species of CoV in mice; also improved survival and induced INF-I. Inhibited CoV in cell lines. Synthetic compound designed for nonstructural proteins.	Wang et al., 2015
	Replication	200 μM	SARS-CoV		
Bananins	Replication	<10 μM	SARS-CoV	Synthetic molecule, highly tolerated, easily produced, but there are no *in vivo* assays	Tanner et al., 2005
Nitazoxanide	Host Enzymes	0.92 $\mu g\ mL^{-1}$	MERS-CoV	Drug already in use to treat viral infections; good inhibition *in vitro*	Rossignol, 2016
Tizoxanide	Host Enzymes	0.83 $\mu g\ mL^{-1}$	MERS-CoV	Drug derived from Nitazoxanide; good inhibition *in vitro*	Rossignol, 2016
Saracatinib	Tyrosine Kinases	2.9 μM	MERS-CoV	Synthetic molecule, highly tolerated, used to treat Alzheimer's disease and easily produced but there are no in vivo assays	Shin et al., 2018
Cyclosporin A (CsA)	Hosts Cyclophilin Family Enzymes	9–32 μM	SARS-CoV, MERS-CoV and MHV	Drug already used to treat several chronic and infectious diseases with broad-spectrum activity among CoVs	de Wilde et al., 2011, 2013; Pfefferle et al., 2011
Alisporivir	Hosts Cyclophilin Family Enzymes	8.3 μM	SARS-CoV	Analog of CsA and has a strong inhibition *in vitro* against SARS-CoV and other CoVs	de Wilde et al., 2017
Interference RNA (iRNAs)	Viral Proteins Translation	70%	SARS-CoV	Different approach, specific targeting of viral proteins; can block replication steps and has no cytotoxicity	Åkerström et al., 2007
	Viral Proteins Translation	99%	SECoV	Different approach, specific targeting of viral proteins; can block replication steps and has no cytotoxicity	Li et al., 2019

EC50: effective concentration of 50%; NE: not evaluated.

TABLE 2 | Ongoing clinical trials of candidate drugs against SARS-CoV-2 in COVID-19 patients.

Drug	Cell culture assays	Inhibition step *in vitro*	Animal assays	Clinical trials	Outcomes in clinical trials	Advantages and/or limitations
Remdesivir	Inhibited SARS-CoV, MERS-CoV, and SARS-CoV-2	Replication (RdRp)	Inhibited EBOV and SARS-CoV in both infected mice and monkeys	Clinical case and clinical trial against SARS-CoV-2	Did not provide antiviral effects or improved clinical outcomes	This is a multicentre, double-blind, placebo-controlled clinical trial, but more studies might be needed to confirm, since this includes 255 people, and the drug has some adverse effects.
Lopinavir and Ritonavir	Inhibited SARS-CoV and MERS-CoV	Replication (protease inhibitor)	NE	Clinical trial with SARS-CoV-2	Did not provide antiviral effects or improved clinical outcomes in severe patients, but, in early infections, clinical outcomes were improved.	This drug combination is used for other human CoVs, but the study was not multicentre, double-blind, and placebo-controlled. More studies are necessary to confirm, since it had only 199 people and the drug showed some adverse effects.
IFN-β	Inhibited SARS-CoV, MERS-CoV, MHV, and HCoV-229E	Host Factors (inducing immune response)	Inhibited SARS-CoV, MERS-CoV, MHV, and HCoV-229E	Clinical trial with SARS-CoV-2 and is used for other diseases	Do not have effect alone	IFN-β is indicated to be safe, with few adverse effects, but in clinical trials, it is only effective when associated with other drugs.
Umifenovir	Inhibited SARS-CoV	NE	NE	Observational study with 81 patients	Did not provide antiviral effects or improved clinical outcomes	This is an observational study and might suffer bias from lack and/or loss of information and data. It is an applicable study, since it demonstrates a tendency, and the drug is already used to treat Influenza viruses.
Corsticosteroids (dexamethasone)	NE	Host factors (controlling immune response)	NE	Clinical trial with 454 treated patients	Reduced death by one-third in invasive mechanical ventilation patients and one-fifth in oxygen without invasive mechanical ventilation patients; however, did not impair mortality in patients without respiratory support	This is a multicentre, double-blind, placebo-controlled clinical trial. More studies are needed to understand better the effect on different phases of COVID-19. May be a good alternative for treating hyperinflammation and hypersecretion of cytokines.
Ivermectin	Inhibited SARS-CoV-2 and arboviruses (CHIKV and DENV)	Replication (nonstructural proteins)	NE	Clinical trials are beginning	NE	Ivermectin is safe for use in humans since it is used to treat several parasitic infections.
Tocilizumab	NE	Inhibitor of IL-6	NE	Ongoing clinical trials with SARS-CoV-2 patients; one with 100 patients concluded.	Positive effects: improved inflammatory markers and decreased the need for ventilatory support in patients	Tocilizumab is already used to treat viral infections, controlling immune response, impairing cytokine storms, improving antiviral response, and providing the best clinical outcomes.
Chloroquine	Inhibited HIV, CHIKV, SARS-CoV, and SARS-CoV-2	Entry	Improved outcomes in FCoV positive cats	Several clinical trials are being conducted	Impairs virus replication and has anti-inflammatory activities	Chloroquine possesses important side effects and is indicated only in severe cases. However, there are some studies with contrasting results regarding its safety, since it can cause arrhythmias, hypoglycemia, neuropsychiatric effects, and depression.

(Continued)

TABLE 2 | Continued

Drug	Cell culture assays	Inhibition step *in vitro*	Animal assays	Clinical trials	Outcomes in clinical trials	Advantages and/or limitations
Hydroxychloroquine	Inhibited HIV, CHIKV, SARS-CoV, and SARS-CoV-2	Entry	NE	Several clinical trials are being conducted	Less toxic option, impairs virus replication	Hydroxychloroquine improved patients' outcomes, including when associated with azithromycin. Less toxic option than chloroquine treatment, but there are studies with contrasting results regarding its safety, since it can cause arrhythmias, hypoglycemia, neuropsychiatric effects, and depression.
Convalescent Plasma	NE	Entry	NE	Case report	Improved outcomes and suppressed viremia.	Administration poses a risk to patients since it is related to donor-dependent variability and compatibility. Antibody titers may interfere with its activity. In addition, it might cause side effects in lung and the cardiovascular system.

EC50: effective concentration of 50%; NE: not evaluated.

The endossomal cathepsins are essential enzymes in viral entry into host cells (Huang et al., 2006), and cathepsin L has been pointed to as playing a crucial role in membrane fusion with the endosomes (Belouzard et al., 2009; Matsuyama et al., 2020). In this context, Shah and coworkers demonstrated the effective activity of tetrahydroquinoline oxocarbazate (CID 23631927), an oxocarbazate inhibitor of cathepsin L, against SARS-CoV. Employing a pseudovirus system with a luciferase reporter to infect 293T cells, the compound inhibited viral entry with an EC_{50} of 273 ηM and CC_{50} > 100 μM (Shah et al., 2010). The authors also showed that the compound CID 23631927 seems to bind with a lower inhibition constant (K_i) to cathepsin L, improving the compound/cathepsin L interaction. This might be related to its optimized structure, with stronger hydrophobic interactions and better hydrogen bonds between the compound and cathepsin L (Shah et al., 2010).

An extensive study screened a library of compounds following Lipinski's rule (Lipinski et al., 2001) and identified three noncytotoxic compounds capable of inhibiting SARS-CoV pseudoparticle entry into 293T cells (Adedeji et al., 2013). *N*-(9,10-dioxo-9,10-dihydroanthracen-2-yl)benzamide (SSAA09E1) blocked early interactions of SARS-CoV S protein with ACE2 (EC_{50} of 6.7 μM and CC_{50} > 100 μM), whereas *N*-[[4-(4-methylpiperazin-1-yl)phenyl]methyl]-1,2-oxazole-5-carboxamide (SSAA09E2) affected cathepsin L activity (EC_{50} of 3.1 μM and CC_{50} > 100 μM). Conversely, [(Z)-1-thiophen-2-ylethylideneamino]thiourea (SSAA09E3) prevented the fusion of the viral envelope with host membrane cells by direct interaction with spike protein (EC_{50} of 9.7 μM and CC_{50} > 20 μM) (Adedeji et al., 2013). The compound SSAA09E3 presented the highest cytotoxic, probably due to the interactions with host proteins. The authors suggested that since these three compounds are derived from molecules with antiviral activities and presented good oral bioavailability and rapid systemic distribution in animal models, they might exhibit interesting pharmacokinetics (Adedeji et al., 2013).

Other compounds also demonstrated to inhibit CoV entry, for example, emodin (6-methyl-1,3,8-trihydroxyanthraquinone), a component from *Rheum officinale* roots, which at 50 μM inhibited the infectivity of S protein-pseudotype retrovirus from SARS-CoV in Vero cells by about 80% (Ho et al., 2007). Besides the entry activity, emodin was described to have an additional post-entry antiviral action. The authors suggested that emodin might be impairing virus release by affecting 3a viral protein, which is related to ion channels in infected Vero cells (Schwarz et al., 2011). This effect may play an important role in immune response.

The exploitation of other natural compounds such as proteins as potential anti-CoV drugs has also been performed. Griffithsin (GRFT) is a protein isolated from the red alga *Griffithsia* sp. that has shown powerful viral entry inhibition against several enveloped viruses, such as the human immunodeficiency virus (HIV). GRFT is capable of binding to terminal mannoses of oligosaccharides and also to glycans localized on the viral envelope glycoproteins (Lusvarghi and Bewley, 2016). GFRT did not present cytotoxicity in Vero cells, human ileocecal colorectal adenocarcinoma cells, human diploid fibroblast cells, and rhesus

monkey kidney cells. Its broad-spectrum antiviral activity *in vitro* was demonstrated against several CoVs such as SARS-CoV (EC_{50} of 0.61 $\mu g\ mL^{-1}$), bovine coronavirus (BCoV) (EC_{50} of 0.057 $\mu g\ mL^{-1}$), MHV (EC_{50} of 0.23 $\mu g\ mL^{-1}$), HCoV-OC43 (EC_{50} of 0.16 $\mu g\ mL^{-1}$), HCoV-229E (EC_{50} of 0.18 $\mu g\ mL^{-1}$), and HCoV-NL63 ($EC_{50} < 0.032\ \mu g\ mL^{-1}$) (O'Keefe et al., 2010). In another study, GRFT inhibited the early stages of MERS-CoV infection in HEK-293T cells (Millet et al., 2016). Furthermore, GRFT improved survival in SARS-CoV-infected mice and protected the Balb/c female mice against infection by binding with S protein (O'Keefe et al., 2010). Altogether, this evidence indicates that GRFT can be considered as a potential SARS-CoV-2 entry inhibitor with activity against S proteins.

Antiviral activity by entry inhibition was also evaluated by employing antibacterial chemotherapeutics. Vancomycin, eremomycin, and teicoplanin glycopeptide compounds used to treat infections caused by Gram-positive bacteria (Preobrazhenskaya and Olsufyeva, 2004), as well as hydrophobic derivatives of these drugs, were described to possess antiviral activity against HIV (Printsevskaya et al., 2005). A study showed that vancomycin, eremomycin, and teicoplanin were not toxic to Vero and T lymphoblast (CEM) cells. Nonetheless, these compounds were not able to inhibit feline CoV (FIPV) and SARS-CoV in assays employing such cell lines. Conversely, the eremomycin derivative molecules labeled 27 and 39 showed the best inhibition profiles against FIPV (EC_{50} of 5.4 and 12 μM, respectively) and SARS-CoV (EC_{50} of 14 and 22 μM, respectively) (Balzarini et al., 2006).

Cationic antimicrobial peptides (AMPs) are another type of peptides that have been considered as potential broad-spectrum antiviral agents. For instance, mucroporin is an AMP found in *Lychas mucronatus* scorpion venom (Dai et al., 2008). Mucroporin was then optimized synthetically, generating mucroporin-M1, which was able to inhibit measles virus (MeV), SARS-CoV, and influenza H5N1. Specifically, mucroporin M-1 affected SARS-CoV pseudovirus entry, with EC_{50} of 14.46 $\mu g\ mL^{-1}$ and CC_{50} of 61.58 $\mu g\ mL^{-1}$, by virucidal activity in HeLa-ACE2 cells (Li et al., 2011). The activity of this synthetic peptide seems to be related to positive charges of the hydrophilic site, which can enhance the interaction with the viral surface, inactivating the viral particle.

Other potential antiviral peptides were selected by Struck and colleges. Through the exploitation of bioinformatics tools, the authors were able to predict sixteen peptides with effective binding onto the receptor-binding domain (RDB) present in S proteins of CoVs. These compounds were then synthesized, and the hexapeptide Tyr-Lys-Tyr-Arg-Tyr-Leu at 14 mM inhibited SARS-CoV and HCoV-NL63 infection in Vero cells without triggering cytotoxicity (Struck et al., 2012). This peptide was designed specifically to bind to the site of interaction with S protein and does not interfere with ACE2 receptor activity, so it might be a good candidate for blocking SARS-CoV-2 entry without impairing host metabolism. Taking into consideration that cellular factors such as the Tumor Necrosis Factor-alpha (TNF-α) converting enzyme (TACE) facilitate SARS-CoV entry (Haga et al., 2008), it is reasonable to suggest that TACE inhibitors could hinder SARS-CoV infection. In this context, TAPI-2, a compound able to inhibit TACE, has shown potent antiviral activity, promoting a 65% blockade of SARS-CoV entry in HEK-293T cells. However, the compound did not affect the virus titer in *in vivo* assays (Haga et al., 2010). The authors suggested that since SARS-CoV attaches to additional receptors such as DC-SIGN and L-SIGN (Jeffers et al., 2004; Han et al., 2007), viral entry might be not be impaired by this molecule.

In addition to amino acid-based inhibitors, monoclonal antibodies (mAbs) have attracted attention due to their use in infectious and chronic disease treatments (Green et al., 2000; Haynes et al., 2009; Pettitt et al., 2013; D'Amato et al., 2014), overcoming drawbacks caused in polyclonal Abs therapy, such as those related to donor compatibility (Marasco and Sui, 2007). Human neutralizing Abs against human CoVs have been generated, targeting S glycoproteins to impair viral entry (Belouzard et al., 2012; Reguera et al., 2012). Notably, several mAbs were identified as inhibitors of MERS-CoV and SARS-CoV infections both *in vitro* and *in vivo*, protecting cells and animals when administered 24 h prior to or post-infection (Lip et al., 2006; Zhu et al., 2007; Agnihothram et al., 2014; Shanmugaraj et al., 2020). The mAbs are developed by merging B lymphocytes and myeloma cells, producing hybridomas capable of recognizing antigens and producing a single Ab class to bind specific epitopes (Lipman et al., 2005). For that reason, mAb cross-reactivity among different coronaviruses seems to be ineffective (Totura and Bavari, 2019). In the particular case of SARS-CoV-2, Wang and coworkers produced mAbs using 51 lineages of SARS-S hybridoma cells and identified 47D11 H2L2-neutralizing Ab through ELISA assays. This antibody was produced using mice cells; therefore, it was further modified to produce a fully human immunoglobulin IgG1, producing the human monoclonal antibody 47D11. The results showed that 47D11 bound to the RBD region and inhibited SARS-CoV-2 entry in Vero cells with an EC_{50} of 0.57 $\mu g\ mL^{-1}$ (Wang et al., 2020a). In this context, this mAb can be used alone or in association with other compounds to treat COVID-19.

Inhibitors of Post-entry Stages of the CoV Replicative Cycle

Among the proteins that are pivotal for CoV viral replication are the main proteases (Mpro) such as the chymotrypsin-like protease (3CLpro) and the papain-like proteases (PPL). These enzymes process viral polyproteins and control replicase complex activity (Anand et al., 2003), figuring as very attractive targets for drug development against CoVs. Several natural products and synthetic peptides have been reported to inhibit Mpro (Cinatl et al., 2005; Vuong et al., 2020).

Gan and coworkers used molecular docking methods to select the octapeptide Ala-Val-Leu-Gln-Ser-Gly-Phe-Arg as Mpro inhibitor of SARS-CoV and evaluated its antiviral activity in infected Vero cells. The octapeptide presented an EC_{50} of 2.7×10^{-2} mg mL^{-1} and a $CC_{50} > 100$ mg mL^{-1}, resulting in a selectivity index of over 3,704 (Gan et al., 2006). Moreover, five Phe-Phe dipeptide inhibitors (A-E) were designed and selected *in silico* to interact with 3CLpro and showed to be able to protect

Vero cells from the cytopathic effect (CPE) caused by SARS-CoV. C analog (JMF1521) was obtained by the condensation of Phe-Phe dipeptide unsaturated ester with cinnamic acid and exhibited the highest activity, with an EC_{50} of 0.18 μM and CC_{50} > 200 μM (Shie et al., 2005). The authors also performed enzymatic assay to evaluate the activity of JMF1521 on 3CLpro and showed that the peptide inhibited the 3CLpro activity with an inhibition constant of 0.52 μM. The results suggested that this analog disposes a rather rigid coplanar structure in the N-terminal motif that results in more effective hydrogen bonds with the enzyme residues (Shie et al., 2005).

Another example of a dipeptide-based compound that can act as a protease inhibitor is dipeptidyl EP128533 (Zhang et al., 2006), which showed antiviral activity against SARS-CoV in Vero cells, with EC_{50} and CC_{50} values of 3.6 and >100 μM, respectively (Zhang et al., 2006). In accordance with that study, it was also demonstrated that EP128533 inhibited SARS-CoV with an EC_{50} of 1.4 μg mL^{-1} and CC_{50} > 100 μg mL^{-1} (Day et al., 2009). However, the compound was not efficient in reducing the effects of viral replication in BALB/c mice (Day et al., 2009). The authors proposed that EP128533 is relatively insoluble and that its lack of activity might be related to a low bioavailability in the animal models.

The dipeptides GC373 (dipeptidyl aldehyde) and GC376 (dipeptidyl bisulfite adduct salt from GC373) were also designed and synthesized as protease inhibitors of the 3CLpro enzyme (Kim et al., 2012). Their activity was assessed *in vitro*, and the results showed that GC373 inhibited HCoV-229E (EC_{50} of 0.2 μM), feline infectious peritonitis virus (FIPV, EC_{50} of 0.3 μM), MHV (EC_{50} of 2 μM), transmissible gastroenteritis virus (TGEV, EC_{50} of 0.3 μM), and bovine coronavirus (BCV, EC_{50} of 0.7 μM) (Kim et al., 2012). GC376 also inhibited HCoV-229E (EC_{50} of 0.15 μM), FIPV (EC_{50} of 0.2 μM), MHV (EC_{50} of 1.1 μM), TGEV (EC_{50} of 0.15 μM), and BCV (EC_{50} of 0.6 μM). The 3CLpro activity of these compounds against SARS-CoV was also analyzed. GC373 and GC376 inhibited enzymatic activity of SARS-CoV 3CLpro, with inhibition constants of 50% of 3.48 and 4.35 μM, respectively (Kim et al., 2012). However, the activity of these compounds was not evaluated using infected cells or animal models. Additionally, the effects of GC373 and GC376 were assessed against feline coronavirus WSU (FCoV-WSU) (EC_{50} values for GC373 and GC376 were 0.15 and 0.40 μM, respectively) (Kim et al., 2013). Moreover, the authors described that concomitant treatment with these compounds can improve the antiviral effect against feline coronaviruses and noted that, since the 3CLpro is conserved among CoVs, it might present broad-spectrum activity (Kim et al., 2013).

RNA-dependent RNA polymerase (RdRp) also figures as a promising target for antivirals. In viral replication, RdRp is responsible for catalyzing the replication of the viral RNA using a complementary RNA as a template. Therefore, compounds that interfere in this process are excellent drug candidates for treating viral infections (Ganeshpurkar et al., 2019). Nucleoside analogs of pyrimidine interfere in uridine triphosphate (UTP) metabolism, directly affecting viral replication (Murphy and Middleton, 2012), as demonstrated by β-D-N^4-hydroxycytidine (NHC), which inhibited SARS-CoV (EC_{50} of 10 μM and CC_{50} > 100 μM) and HCoV-NL63 (EC_{50} of 400 nM and CC_{50} > 100 μM) (Barnard et al., 2004). NHC presented a potent antiviral activity against SARS-CoV-2 in infected Vero (IC_{50} of 0.3 μM and CC_{50} of > 10 μM) and Calu-3 cells (IC_{50} of 0.08 μM and CC_{50} > 100 μM) (Sheahan et al., 2020b). The authors assessed the broad-spectrum antiviral activity of NHC against MERS-CoV (IC_{50} 0.024 μM) and SARS-CoV (IC_{50} 0.14 μM) (Sheahan et al., 2020b) and also evaluated the NHC effect in SARS-CoV- and MERS-CoV-infected mice. NHC improved pulmonary function and decreased viral load in lung, and the authors proposed that NHC might be useful for emerging CoVs. Another pyrimidine analog with potential antiviral activity is 6-azauridine, which inhibited HCoV-NL63 replication in LLC-MK2 cells with an EC_{50} of 32 nM and CC_{50} of 80 μM (Pyrc et al., 2006).

Ribavirin is a synthetic nucleoside analog of guanosine used for the treatment of patients chronically infected by the hepatitis C virus (HCV) (PubChem, 2005c). The antiviral activities of ribavirin against several RNA viruses have been described, and it also presents broad-spectrum antiviral activities for CoVs (Chan et al., 2013; Shen et al., 2016). Its activities were described for SARS-CoV *in vitro* (EC_{50} of 20 μg mL^{-1} and CC_{50} > 200 μg mL^{-1}) (Saijo et al., 2005). Nevertheless, no viral load reduction was observed *in vivo* when employing BALB/c mice (Barnard et al., 2006). The *in vitro* decrease of ribavirin efficacy was demonstrated to be associated with the excision of its nucleoside analogs by conserved coronavirus proofreading mechanisms (Ferron et al., 2017). Moreover, ribavirin showed good results for the treatment of critical MERS-CoV patients (Al-Tawfiq et al., 2014), and the combined treatment of ribavirin with type I Interferons (IFN-I) in primate models improved MERS disease symptoms (Falzarano et al., 2013b). Although ribavirin has been given as part of treatment regimens for SARS and MERS patients, meta-analyses of cases of study have found limited efficacy of its activities in treating patients with highly pathogenic coronavirus respiratory syndromes (Morra et al., 2018).

What is more, a nucleoside analog based on the acyclic sugar scaffold of acyclovir showed antiviral potential against coronaviruses (Tan et al., 2004). Peters and contributors demonstrated that this compound has powerful antiviral activity against MERS-CoV (EC_{50} and CC_{50} of 23 and 71 μM, respectively) and HCoV-NL63 (EC_{50} and CC_{50} of 8.8 and 120 μM, respectively) (Peters et al., 2015). However, the authors did not suggest mechanisms by which this analog impairs viral replication, leaving open to question whether it acts like its precursor acyclovir, impairing viral replication or by an alternative mechanism of action.

In terms of other drug options for the post-entry stages of the viral replicative cycle, it is possible to report the activities of Niclosamide, a drug used in antihelminthic treatment (Katz, 1977). Niclosamide presented antiviral activity on post-entry steps of SARS-CoV infection in Vero cells, with an EC_{50} of 1–3 μM and CC_{50} of 250 μM (Wu et al., 2004). Similarly, this compound suppressed the cytopathic effect of SARS-CoV at a concentration <1 μM and inhibited viral replication with an EC_{50} value of less than 0.1 μM in Vero E6 cells (Wen et al., 2007). Both authors suggested that Niclosamide impairs

post-entry steps. However, this effect seems to not be related to an interaction with 3CLpro.

An additional potential compound is mycophenolic acid (MPA), an antibiotic derived from penicillium fungal species (PubChem, 2005b), which inhibited MERS-CoV replication in Vero cells with an EC_{50} of 2.87 μM (Hart et al., 2014). However, MPA was not active against SARS-CoV in either *in vitro* or *in vivo* assay (Barnard et al., 2006). The data suggested that MPA inhibits the enzyme IMP dehydrogenase, inducing apoptosis on alveolar macrophages and consequently inhibiting or suppressing cellular immune responses that are important for preventing or limiting viral infection (Barnard et al., 2006).

Bananins, on the other hand, are a class of adamantane-based compounds conjugated with a pyridoxal moiety (vitamin B6) (Kesel, 2003). These molecules showed effective inhibition of SARS-CoV in FRhK-4 cells, with EC_{50} < 10 μM and CC_{50} of 390 μM. On the basis of both time addition and ATPase assays, the authors proposed that the action of bananin is mainly on the post-entry step of virus replication and may be related to an effect on the helicase function and/or on components of cellular pathways (Tanner et al., 2005).

Finally, the nonstructural protein 10 (nsp10) of CoVs was described as being responsible for a stimulatory effect on nsp16, a classical S-adenosylmethionine-dependent (nucleoside-2'-*O*)-methyltransferase that acts in RNA binding or catalysis. The peptide TP29 was designed as a ligand to MHV nsp10 and presented broad-spectrum activity, inhibiting SARS-CoV (EC_{50} of 200 μM) and MHV (EC_{50} of 60 μM) replication in infected cell lines (Wang et al., 2015). The authors also assessed TP29 activity in MHV infected mice and demonstrated that treatment improved survival, decreased viral load in liver, and induced type 1 IFN. Based on these data, it was suggested that TP29 impaired nsp10/nsp16 2'-*O*-MTase activity, dysregulating the genome replication process.

Looking Toward Host Machinery: A Different Approach to CoV Treatment

Targeting the host process during viral infection figures as a promising alternative for drug development and can play an important role in abrogating viral replication (Sayce et al., 2010; Ullah et al., 2019). Nitazoxanide is a broad-spectrum antiviral agent exploited for the treatment of, for instance, influenza A and B viruses, as well as Ebola virus (EBOV) (Rossignol, 2014; Jasenosky et al., 2019), with its activity related to the interference in host-regulated pathways during viral replication (Rossignol, 2016). *In vitro* studies demonstrated that Nitazoxanide was able to inhibit MERS-CoV in LLC-MK2 cells, with an EC_{50} of 0.92 μg mL^{-1}. The authors suggested that nitazoxanide affects pro-inflammatory cytokines and suppresses their overproduction (Rossignol, 2016).

Another host-target compound is Saracatinib (AZD0530), a tyrosine kinase (SFK) inhibitor. This compound suppressed the early stages of the MERS-CoV replicative cycle in Huh7 cells (EC_{50} of 2.9 μM and CC_{50} > 50 μM), possibly by affecting the SFK pathways (Shin et al., 2018). SFK possesses a central function in signaling pathways such as ERK/MAPK and PI3K/AKT (Thomas and Brugge, 1997), which are strictly related to CoV infection. Therefore, SFK inhibition might promote viral clearance and can be used in association with other drugs (Shin et al., 2018).

Moreover, Cyclosporin A (CsA), a peptide with activity on the cyclophilin family of host enzymes (isomerases that act as chaperones) (PubChem, 2005a; Davis et al., 2010), inhibited SARS-CoV (100% inhibition at 16 μM), HCoV-229E (75% inhibition at 16 μM), and MHV (100% inhibition at 16 μM) in human and animal infected cell culture. CsA presented broad-spectrum antiviral activity against CoVs, and it seems to interfere with genome replication/transcription during CoV infections (de Wilde et al., 2011, 2013; Pfefferle et al., 2011). Alisporivir, a non-immunosuppressive cyclosporin A analog, inhibited the replication of SARS-CoV in Vero E6 infected cells at low-micromolar concentrations (EC_{50} of 8.3 μM; CC_{50} > 50 μM). This compound also showed broad-spectrum anti-CoV activity, inhibiting MERS-CoV EMC/2012 (EC_{50} of 3.6 μM), MERS-CoV N3/Jordan (EC_{50} of 3 μM), and SARS-CoV MA-15 (EC_{50} of 1.3 μM) *in vitro* (de Wilde et al., 2017). However, the authors demonstrated that Alisporivir did not enhance survival in CoV-infected mice (de Wilde et al., 2017).

Other biomolecules that are promising as drug antivirals are interference RNAs (iRNAs). These macromolecules are small non-coding RNAs associated with controlling the expression of genetic information (Wilson and Doudna, 2013) and have been described as promising candidates for the treatment of hepatitis B virus (HBV), HCV, HIV, and human T-cell lymphotropic virus (HTLV) infections (Ma et al., 2007; Shah and Schaffer, 2011; Sanan-Mishra et al., 2017). Short interference RNAs (siRNAs) were described as being effective for *in vitro* antiviral treatment of FIPV, a type of FCoV (McDonagh et al., 2011, 2015). Most recently, Li and colleagues designed and synthesized siRNAs that targeted the M and N genes of swine and porcine coronaviruses (SECoV and PDCoV, respectively). These siRNAs inhibited up to 99% of the expression of these proteins in both Vero and LLC-PK1 infected cells (Li et al., 2019). Additionally, synthetic siRNAs targeting the structural proteins E, M, and N of SARS-CoV have also been developed and showed reductions of the target gene expressions in Vero cells (Shi et al., 2005). Moreover, siRNAs targeting the structural proteins 7a, 7b, 3a, 3b, and S reduced SARS-CoV progeny in Vero cells by approximately 70% (Åkerström et al., 2007). The different authors propose that treatment with siRNAs can improve treatment-resistance among viruses and that these molecules can be designed to target multiple proteins, aiming at broad-spectrum activity.

Ongoing Clinical Evaluations With Candidate Drugs Against SARS-CoV-2

The current situation of COVID-19 pandemic has accentuated the urgency of the demand for effective treatments. Based on previous data concerning activities against other viruses and empirical knowledge from treatments used in case reports, several drugs have entered clinical trial phases to access their therapeutic potential against SARS-CoV-2. In this section, we discuss the current knowledge on the most promising candidates

for the treatment of COVID-19. Data for these drugs are summarized in **Table 2**.

The nucleoside analog Remdesivir (GS-5734) is a monophosphoramidate prodrug that has been described as having antiviral activity against the EBOV in non-human primates (Warren et al., 2016, 57). Its activity was assessed in human airway epithelial (HAE) cells infected with SARS-CoV (EC_{50} of 0.069 μM and CC_{50} > 10 μM) and MERS-CoV (EC_{50} of 0.074 μM and CC_{50} > 10 μM) and was demonstrated to inhibit RdRp of these viruses. Also, GS-5734 reduced infectious virus production of bat CoV by 1.5 to 2.0 log_{10} in HAE cells and reduced virus titers and virus-induced lung pathologies in a SARS-CoV assay *in vivo* (Sheahan et al., 2017). This compound also reduced the severity of MERS-CoV disease, virus replication, and damage in the lungs of rhesus macaques (De Wit et al., 2020). The clinical efficacy of GS-5734 has been assessed by several clinical trials in different countries like France (NCT04365725), Canada (NCT04330690), and the United States (NCT04292899), which have been conducted based on the first reported treatment of COVID-19 with Remdesivir in Washington, United States (Holshue et al., 2020). In the first findings from Wang and coworkers, which were from a randomized, double-blind, multicenter, and placebo-controlled trial with 255 patients, Remdesivir did not present significant antiviral effects against SARS-CoV-2, nor did it improve clinical outcomes (Wang et al., 2020d). To date, there are several active clinical trials registered in the PubMed database involving this compound. However, most of them presented no conclusive outcomes.

Another two candidates are Lopinavir and Ritonavir, which are protease inhibitors used in association to treat HIV infections (Cvetkovic and Goa, 2003; Mills et al., 2009). Lopinavir demonstrated antiviral activities, protecting cells from MERS-CoV infection (EC_{50} of 8 μM) and reducing viral loads in animal assays (de Wilde et al., 2014; Kim et al., 2015). Ritonavir also demonstrated anti-MERS-CoV activities with an EC_{50} of 24.9 μM (Sheahan et al., 2020a). It is important to point out that these results do not agree with another work that was unable to demonstrate *in vitro* antiviral activity of Lopinavir against MERS-CoV (Chan et al., 2013). In clinical assays for MERS-CoV, the association of Lopinavir with Ritonavir reduced adverse clinical outcomes and viral load in infected patients (Sheahan et al., 2020a; Yao et al., 2020a). In particular, for SARS-CoV, Lopinavir and Ritonavir presented a low to medium antiviral activity *in vitro*, and *in vivo* assays have not been performed yet (Yao et al., 2020a). In addition, Lopinavir and Ritonavir played an important role in the clinical outcome of SARS-CoV-infected patients by reducing symptoms and the period of hospitalization, representing a possibility for the treatment of SARS-CoV-2 (Chu et al., 2004). Cao and collaborators conducted a randomized clinical trial with 199 patients with severe COVID-19 (Cao et al., 2020b). Treatment of the patients with the association Lopinavir/Ritonavir did not improve symptoms, nor impaired detectable viral RNA when compared to standard care (supplemental oxygen, noninvasive and invasive ventilation, antibiotic agents, vasopressor support, renal-replacement therapy, and extracorporeal membrane oxygenation). Additionally, the treatment generated relevant adverse effects in some of the patients (Cao et al., 2020b). The authors proposed that the low efficacy of Lopinavir with Ritonavir might be associated with the time of administration, since individuals that were treated at the onset of the disease had improved clinical results (Cao et al., 2020b). Later, it was shown that the association of lopinavir and ritonavir with interferon-β1 and ribavirin to treat mild to moderate COVID-19 patients alleviated symptoms and decreased the durations of viral infection and hospital stay (Hung et al., 2020). This might be related to their inducing cellular immune response, impairing virus replication.

The type 1 interferons (IFN-I) have also been employed in clinical trials. These proteins belong to the cytokine family and are associated with the immune response in viral infections, thus playing major roles in antiviral immunity due to their immunomodulatory properties (Samuel, 2001). Therefore, they are commonly employed in the treatment of several diseases such as Hepatitis C (Kobayashi et al., 1993). There are two subtypes of IFN-I, alpha (IFN-α) and beta (IFN-β) (Samuel, 2001). IFN-β is associated with more potent activity (Chan et al., 2015) and is therefore capitalized on in the treatment for multiple sclerosis patients (Axtell et al., 2010). Due to its more potent inhibition profile, it was associated with potent antiviral effects against SARS-CoV, MERS-CoV, MHV, and HCoV-229E *in vitro* and *in vivo* (Sperber and Hayden, 1989; Vassão et al., 2000; Hensley et al., 2004; Falzarano et al., 2013a; Chan et al., 2015). IFN-β, in particular, has a protective effect in endothelial cells, up-regulating CD73 and consequently stimulating the anti-inflammatory molecules and maintenance of endothelial barrier (Bellingan et al., 2014; Sallard et al., 2020). However, a clinical trial with 301 patients showed that this effect was not sufficient to decrease mortality in SARS patients (Ranieri et al., 2020). Therefore, in SARS-CoV-2, IFN-β has been associated with other drugs in clinical trials, improving outcomes in COVID-19 patients as in lopinavir or ribavirin (Hung et al., 2020).

COVID-19 patients with mild to severe symptoms can develop hyperinflammation and hypercytokinaemia, which can lead to multiple organ failure and death (Mehta et al., 2020). The employment of corticosteroids has shown to be an alternative for overcoming the cytokine storm and hyperinflammation due to its activities on immune cells (Wilkinson et al., 1991). Such a capitalization was previously reported in SARS-CoV patients during the 2002–2003 epidemic (Chihrin and Loutfy, 2005). For SARS-CoV-2, corticosteroids can improve the clinical condition of patients, reducing hyperinflammation and the development of ARDS, with faster improvement of symptoms (Wang et al., 2020c; Zha et al., 2020). However, contrasting data concerning the efficacy of these drugs was described recently, showing that corticosteroids did not improve symptoms in COVID-19 patients (Zha et al., 2020). Moreover, dexamethasone emerged as a potential drug for treating COVID-19 patients, as shown by the results of a randomized, controlled, open-lab, and multicenter trial that assessed the effects of dexamethasone in 454 patients, described to date in pre-print findings (Horby et al., 2020). Data suggested that dexamethasone reduced death in one-third of patients in invasive mechanical ventilation and one-fifth of patients in non-invasive oxygen mechanical ventilation. However, it did not impair mortality in patients with no respiratory support (Horby et al., 2020). Other trials

have been conducted, such as NCT043274011, but considering the preliminary results, the WHO suggested that treatment with dexamethasone may be applied during the third phase of COVID-19, when the hyperinflammation is determined, and respiratory support is needed.

Another antiviral drug assayed toward SARS-CoV-2 is Umifenovir, a licensed antiviral exploited for the prophylaxis and treatment of influenza viruses (Arbidol), which demonstrated good pharmacokinetics when absorbed by the organism (Proskurnina et al., 2020). This drug has an antiviral effect against SARS-CoV *in vitro* at 50 $\mu g\ mL^{-1}$ (Khamitov et al., 2008). Lian and coworkers coordinated an observational study with 81 patients with moderate to severe SARS-CoV-2 infection (Lian et al., 2020) that demonstrated that Umifenovir neither shortened the hospitalization period nor improved prognosis in infected patients (Lian et al., 2020).

Broad-spectrum drugs used against parasitic infections such as Ivermectin (Campbell, 2012; Laing et al., 2017) have also been investigated due to their antiviral activity against Dengue virus (DENV), Influenza A viruses, Chikungunya virus (CHIKV), and HIV (Tay et al., 2013; Götz et al., 2016; Varghese et al., 2016; Caly et al., 2020). The activity of Ivermectin is based on impairing several stages of viral replication, for instance, interfering with nonstructural proteins (Varghese et al., 2016). Caly and collaborators assessed the effect of Ivermectin on SARS-CoV-2 replication in Vero cells, showing that, at 5 μM, the compound presented no toxicity to cells and inhibited up to 99% of viral replication by a possible antiviral effect on viral release, which is consistent with previous data on its activity against other RNA viruses (Tay et al., 2013; Caly et al., 2020). Clinical trials have been conducted in different medical centers in Argentina (NCT04381884), Mexico (NCT04391127), Spain (NCT04390022), and the United States (NCT04374279) to assess the clinical implications of the use of Ivermectin for COVID-19. However, to the best of our knowledge, there are no published results on this topic. NCT04343092, a phase 1 clinical trial in Iraq, was conducted to its completion and evaluated the efficacy of Ivermectin in COVID-19 patients, so the results might be published soon.

According to Guan and colleagues, approximately 15.7% of Chinese patients with COVID-19 developed severe pneumonia and cytokine release syndrome (CRS), an important factor leading to rapid progression of the disease (Chousterman et al., 2017; Guan et al., 2020). In this context, one of the key cytokines involved in infection-induced cytokine storm is interleukin 6 (IL-6) (Scheller and Rose-John, 2006; Zhang et al., 2020a). Tocilizumab is an IL-6 receptor antagonist approved by the US FDA for the treatment of severe CRS (Grupp et al., 2013) and figures as an interesting drug to treat the cytokine storm caused by SARS-CoV-2 (Zhang et al., 2020b). The treatment of patients with severe COVID-19 with Tocilizumab presented no complications in the 21 assisted patients, with an average age of 56.8 ± 16.5 and no history of illness deterioration or death. Thus, it immediately improved the clinical outcome and appeared to be an effective treatment for reducing mortality (Xu et al., 2020). Another study employing the treatment of COVID-19 patients with Tocilizumab for 14 days reinforced these observations. The treatment was observed to cause an effective decrease in inflammatory markers, radiological improvement, and a reduction in ventilatory support requirements for these patients (Alattar et al., 2020). Additionally, Toniati and collaborators administered Tocilizumab in 100 patients in Italy (average age of 62 years old) who had been diagnosed with COVID-19 pneumonia and ARDS and required ventilatory support. Overall, at 10 days of follow-up, the respiratory condition was improved or stabilized in 77% of the patients, and, based on these data, the response to this drug in patients with severe COVID-19 was rapid, sustained, and associated with significant clinical improvement (Toniati et al., 2020).

Chloroquine is a 9-aminoquinole that increases the pH in acidic vesicles (Mauthe et al., 2018) and possesses antiviral activities against HIV and other viruses (Jacobson et al., 2016; Al-Bari, 2017). Chloroquine was described as an entry inhibitor of SARS-CoV infection in Vero cells and prevented cell-to-cell spread of the virus (Vincent et al., 2005). Furthermore, it affected the entry and post-entry stages of the replicative cycle of FCoV in *Felis catus* cells and monocytes. Additionally, an *in vivo* study in cats demonstrated that treatment with chloroquine improved the clinical score of treated groups when compared to the untreated group (Takano et al., 2013). Chloroquine also had its anti-CoV activities tested in Vero cells (EC_{50} of 5.47 μM) (Wang et al., 2020b; Yao et al., 2020b). Despite the performance of chloroquine *in vitro*, clinical studies conducted in China and France showed contradictory clinical data (Chen J. et al., 2020; Chen Z. et al., 2020; Gao et al., 2020; Molina et al., 2020). Gao and collaborators indicated that chloroquine phosphate was recommended to treat COVID-19-associated pneumonia only during urgent clinical demand because of its antiviral and anti-inflammatory activities (Gao et al., 2020). Hydroxychloroquine is an analog of chloroquine that was described as having antiviral activity, inhibiting SARS-CoV-2 *in vitro* with an EC_{50} of 0.72 μM (Liu et al., 2020; Yao et al., 2020b). In clinical trials, an open-label non-randomized study by Gautret and colleagues affirmed that hydroxychloroquine reduced symptoms from SARS-CoV-2 patients and that association with azithromycin could reinforce its effects (Gautret et al., 2020). However, these results have been questioned. The study had a small sample size, and there were limitations in the methodologies (Juurlink, 2020).

Recent studies have been contradicting the safety of chloroquine and hydroxychloroquine use, as these drugs presented severe side effects that interfered with their clinical use, even during short-course therapies (Juurlink, 2020; Liu et al., 2020). Apart from the mild adverse effects, such as pruritus, nausea, and headache, these drugs can predispose patients to life-threatening arrhythmias, an effect that may be enhanced by concomitant use of azithromycin (Chorin et al., 2020). Both chloroquine and hydroxychloroquine interfere with ventricular repolarization, leading to prolongation of the cardiac QT interval and an increased risk of torsades de pointes (TdP), which is a risk especially for patients with cardiac disease, for children, or for those taking other drugs that delay repolarization (Mzayek et al., 2007; Pukrittayakamee et al., 2014; Juurlink, 2020; Ursing et al., 2020). Others possible types of damage are hypoglycemia, even in non-diabetic patients

(Unübol et al., 2011; El-Solia et al., 2018); neuropsychiatric effects, including agitation, insomnia, confusion, paranoia, depression, psychosis, and suicidal ideation (Mohan et al., 1981); hypersensitivity reactions, such as severe cutaneous adverse reactions (Cameron et al., 2014; Girijala et al., 2019); and drug–drug interactions, which are improved by genetic variability (genetic polymorphisms of hepatic cytochrome P450 enzyme 2D6 (CYP2D6), responsible for chloroquine metabolization) (Kirchheiner et al., 2008; Lee et al., 2016). There is a lack of reliable information on target concentrations or doses for COVID-19, and so doses that proved effective and safe in malaria for both adults and children are considered for the treatment (Smith, 2020). Recently, the WHO stopped the hydroxychloroquine arm of the Solidarity trial to treat COVID-19 based on an absence of effectiveness in reducing the mortality of hospitalized COVID-19 patients (WHO, 2020c). Besides, the FDA also cautioned against the administration of hydroxychloroquine or chloroquine in COVID-19 patients, mainly due to the risk of heart rhythm issues (FDA, 2020). From these results, it is evident that the use of these drugs for COVID-19 requires further investigation.

An alternative treatment for COVID-19 is the utilization of convalescent plasma (CP) (Chen L. et al., 2020). This treatment refers to plasma therapy based on plasma or plasma derivatives, obtained from donors who were previously infected and have developed antibodies. This plasma/derivative is, in its turn, transfused into individuals with acute SARS-CoV-2 infection (Garraud, 2017; Cao and Shi, 2020). Even though the mechanism of action of convalescent plasma therapy is not fully understood, it presented great results in the treatment of patients with SARS during the SARS-CoV outbreak in Hong Kong in the early 2000s (Cheng et al., 2005). It is possible that the efficacy of CP therapy is due to the fact that the antibodies from convalescent plasma might suppress viremia (Chen L. et al., 2020). Duan and colleagues reported CP transfusion to rescue ten severe cases of SARS-CoV-2 adult patients. The study showed that one dose (200 mL) of CP significantly increased or maintained the neutralizing antibodies at a high level, leading to the disappearance of viremia in 7 days. Clinical symptoms rapidly improved within 3 days, and radiological examination showed varying degrees of absorption of lung lesions within 7 days. According to these results, CP can also provide a promising rescue option for severe COVID-19 (Duan et al., 2020). However, the author suggested key points to guarantee the effectiveness of CP therapy: Ab titers and the treatment time point. Firstly, taking into consideration previous knowledge from MERS-CoV CP therapy, Abs in plasma donor must have a titer equal or higher of 1:80 (Ko et al., 2018). This titer is only found in recently recovered patients, since antibody levels decrease 4 months after the disease. Secondly, patients receiving CP treatment prior to 14 days post-infection responded better than patients treated after 14 days (Duan et al., 2020).

PERSPECTIVES

This review aimed to summarize and discuss data from the literature regarding compounds that possess anti-CoVs activities and that could be further exploited for the treatment of human and animal CoVs. Furthermore, we described ongoing clinical trials for SARS-CoV-2 in order to elucidate the current findings and discussed the relevant features concerning candidate drugs against SARS-CoV-2.

As previously mentioned, most human-related CoVs emerged by zoonotic transmission from animals (Huynh et al., 2012; Coleman and Frieman, 2014; Reusken et al., 2016). Since *Coronaviridae* seem to have a very well conserved genome and structures among their viruses (Huentelman et al., 2004a; Guan et al., 2012; Yang and Leibowitz, 2015; Madhugiri et al., 2018), it is possible to hypothesize that compounds with antiviral activities against different human and/or animal CoVs (broad-spectrum activity) could be potential candidates for SARS-CoV-2 treatment. In a less optimistic scenario, the chemical structures of such compounds and their pharmacological outcomes have the potential to set some light on the drug design of possible anti-SARS-CoV-2 drugs.

Among the strategies for drug design, targeting host-immune factors or using iRNAs figure as promising alternatives for antiviral drug development. Also, the exploitation of *in silico* studies for drug screening to seek specific targets, as well as for a better comprehension of their interactions with viral biomolecules, has been shown as a promising tool for expediting drug development. By narrowing down the number of drug candidates, *in silico* studies have the potential to avoid the laborious and generally costly synthesis of many of these compounds (Lengauer and Sing, 2006; Villegas-Rosales et al., 2012). Nevertheless, several predicted compounds in the literature have only been screened by *in silico* and/or interaction assays (Chen et al., 2005; Kaeppler et al., 2005; Lee et al., 2005; Kim et al., 2012; Arya et al., 2020; Balasubramaniam and Reis, 2020), which ultimately hinders the proper assessment of the antiviral activities of the compounds. Therefore, it is imperative that these studies be associated with *in vitro* and *in vivo* assays in order to confirm the predicted activities in biological models and also to evaluate pharmacological outcomes (National Research Council (US) Committee on Applications of Toxicogenomic Technologies to Predictive Toxicology, 2007). Therefore, this review encompassed only compounds that have been evaluated by, at least, *in vitro* models (**Table 1**).

In this context, from the molecules and drugs described as having *in vitro* activity, we highlighted the most promising to suggest further evaluation using *in vivo* systems of CoV infection, especially SARS-CoV-2 infection. The compounds are: NAAE, Glycyrrhizin, 2-acetamido-α-D-Glucopyranosylamine derivative, Tetrahydroquinoline oxocarbazate (CID 23631927), SSAA09E1, 2 and 3, Emodin, Eremomycin 27 and 29, Mucroporin-M1, Monoclonal antibody 47D11, AVLQSGFR, Phe-Phe dipeptide inhibitor C (JMF1521), GC373 and 376, 6-azauridine, Acyclic sugar scaffold of acyclovir, and Bananins. As described above, these compounds were capable of significantly impairing CoV infection in cell cultures and might enable important progress into the treatment of described CoVs as well as viruses that might be responsible for future viral outbreaks.

Here, we also described compounds that were evaluated *in vivo* to elucidate their role in the pathogenesis of CoVs

as well as to assess possible adverse effects. It is important to emphasize that there is a lack of *in vivo* model assays, representing a delay in anti-CoV drug development, which directly impacts the SARS-CoV-2 pandemic. Here, we identified some studies that employed animal models, such as in Balb/c mice and C57BL/6, to evaluate the antiviral effect of compounds in CoV infection (Cinatl et al., 2003; Saijo et al., 2005; Barnard et al., 2006; Zhang et al., 2006; Day et al., 2009; Hart et al., 2014). The *in vivo* assays allow the gathering of knowledge regarding the ADMeTox profile of these compounds in complex biological systems, the viral titers in different organs, host immune responses to the infection, and also potential tissue damage caused by the viruses in the presence or absence of candidate drugs, which represents an advance in understanding pathologies caused by viral infections (Adachi and Miura, 2014). It is also important to emphasize that protocols used in studies of animal-related viruses are not easily translated onto human CoVs, since these viruses are classified to different biological safety levels, representing a risk of infection to scientists (Bayot and King, 2020; CDC, 2020b). Additionally, the pathologies induced by animal CoVs are mostly related to gastrointestinal symptoms, differently to what is observed for human-related CoVs, which mostly affect the upper respiratory system (Pedersen et al., 1984; Coleman and Frieman, 2014). The development of refined and secure protocols to study SARS-CoV-2 infection and its treatment options is required. Bearing in mind the obstacles cited above, assessment of the effect in animal models and further translation to humans remains one of the main challenges.

However, some of the studies were able to assess the antiviral effects of some compounds *in vivo*. The most relevant compounds we propose that may represent immediate candidates to clinical trials, considering the urgency of COVID-19, are Griffithsin (GRFT), β-D-N^4-hydroxycytidine (NHC), TP29, Cyclosporin A (CsA), Alisporivir, iRNAs, Saracatinib, Tizoxanide, Nitazoxanide, Niclosamide, and Ribavirin. These compounds abrogated CoV infection *in vitro* and *in vivo* and improved the symptoms and survival of animals. In addition, Saracatinib, Tizoxanide, Nitazoxanide, Niclosamide, and Ribavirin are molecules licensed to treat diseases such as those from viral and helminthic infections or Alzheimer's disease, representing possibilities for clinical trials as repurposed drugs.

Regarding clinical trials, most drugs discussed in this review presented adverse effects such as nausea, headache, diarrhea, urticaria, pathologies related to the gastrointestinal system, and interference with liver enzymes (Ruiz-Irastorza et al., 2010; Takano et al., 2013; Roques et al., 2018; Yao et al., 2020a). Remdesivir, Lopinavir and Ritonavir, and Umifenovir are drugs employed for the treatment of other viral infections such as EBOV and SARS-CoV, but, in the clinical trials with COVID-19 patients, these treatments did not reduce symptoms and/or decrease viral load. Tocilizumab, Chloroquine, and Hydroxychloroquine have been demonstrated to inhibit SARS-CoV-2 *in vitro* and, in some clinical trials, reduced COVID-19 symptoms, the period of hospitalization, and the viral load in patients despite the strong adverse effects of Chloroquine (**Table 2**). Even so, recent studies are contradicting the safety profiles of Chloroquine and Hydroxychloroquine, since they might cause arrhythmia in patients, representing risk for a considerable number of patients (Juurlink, 2020).

Ongoing studies have been evaluating IFN-β and Ivermectin as treatments against COVID-19. IFN- β can be associated with other drugs, collaborating to control immune response against the viral infection (**Table 2**). On the other hand, corticosteroids, such as dexamethasone, sound promising, but there are some issues related to their use. These compounds induce immunosuppression and, when administered during initial phases (viral replication), might dysregulate T-cell production and activation of B cells for antibody secretion, which are essential for viral clearance (Cohn, 1991; Giles et al., 2018). Furthermore, convalescent plasma therapy is an alternative approach that presented positive effects in studies on SARS-CoV-2/COVID-19 patients. However, its safety is not well defined due to donor-dependent variability and compatibility (antibody titers and other factors vary among donors), which might cause severe adverse effects in lung and cardiovascular system and, in some cases, may even transmit diseases (Roback and Guarner, 2020).

Despite the finding regarding these drugs, it is important to take some aspects into consideration: i) the trials were generally conducted with a significant number of patients in each study, but potentially not enough to expand the results to public healthcare; ii) some of the studies were observational, which means they were based on public data that may not be well documented, leaving information gaps about particular health issues; additionally, the outcomes in patients are defined by their own circumstances, and not by an investigator; iii) some studies were not placebo-controlled and double-blind, so the placebo effect cannot be discarded (Kernan et al., 1999; Hess and Abd-Elsayed, 2019); iv) the trials were conducted by selecting a group of COVID-19 patients, considering mild, moderate or severe cases, and different outcomes can be expected in each situation since viral load, the progression of the disease, and immune response are additional factors (Kernan et al., 1999; Hess and Abd-Elsayed, 2019). Therefore, drugs with no effect in severe cases cannot be rejected as a possible treatment in mild to severe cases. When these aspects are not considered, the investigators might be open to commiting type I or II error in trials (Kernan et al., 1999; Hess and Abd-Elsayed, 2019). For that matter, it is also important to consider that SARS-CoV-2 is a new virus and that we currently have limited knowledge about its physiopathology. Finally, the development of new treatment options is critical, and efforts have been focused on targeting therapies that aim to improve patient outcome by increasing antiviral activity associated with minimal toxicity.

Another point to be considered in CoV treatment is that RNA viruses are known to have high levels of mutations (error rate) in the replication process (Ganeshpurkar et al., 2019). This can result in resistance to antiviral treatment, as observed for HIV, HCV, and Influenza viruses (Laplante and St George, 2014; Li and Chung, 2019; Olearo et al., 2019; Takashita, 2020). A recent study in pre-print pointed to the genomic variability of SARS-CoV-2 and the intra-patient capacity of polymorphic quasispecies, which may offer resistance to antiviral drugs (Karamitros et al., 2020). In addition, previous studies demonstrated that the

use of Chloroquine analogs for decades against malaria has established chloroquine-resistant Plasmodium strains (Stocks et al., 2002; Al-Bari, 2017; Aguiar et al., 2018). Due to the beneficial immunomodulatory effects of analogs on the severe inflammatory complications of several viral diseases, such as HIV and SARS-CoV infections, these drugs have been tested indiscriminately (Jacobson et al., 2016; Al-Bari, 2017). However, there is a possibility that prophylactic exposure to pro-apoptotic chloroquine drugs caused natural selection for strains of viruses and other parasites that have enhanced anti-apoptotic abilities (Parris, 2004). Despite the side effects, the wide use of some drugs during the SARS-CoV-2 pandemic might raise concerns regarding the emergence of resistant viral strains in the future, and we emphasize the lack of information on the resistance associated with these drugs in the treatment of viral infections.

CONCLUSION

The spread of SARS-CoV-2 worldwide is classified as a pandemic and represents a threat to global public health. By July 4, 2020, SARS-CoV-2 had infected 10,922,324 people and had caused 523,011 deaths around the world (WHO, 2020b). In this context, compounds described to possess antiviral activity against human and/or animal coronaviruses could provide relevant information for the development of novel SARS-CoV-2 treatments. Herein, we presented and discussed the most promising compounds that can figure as possible candidates for clinical trials. Moreover, ongoing clinical trials evaluating possible COVID-19 therapies were also highlighted.

From what was presented in this review, a plethora of different potential compounds can be capitalized as possible drugs or even set points for further drug development seeking to mitigate the SARS-CoV-2/COVID-19 outbreak. However, time, resources, and new experimental protocols are essential for advancing an efficacious treatment. In addition, and despite the urgency of treatment protocols, it is important to point out the striking need for the establishment of fail-proof regulatory initiatives that could prevent impacts on the healthcare of patients that could, otherwise, be avoided by a more stringent control.

In this context, this review describes drugs that might be overlooked for future analysis and could possibly become effective antiviral treatments. As a final remark, we conclude that, to date, there is no "one hundred percent" effective antiviral therapy against SARS-CoV-2/COVID-19 and that further research is needed to achieve the best therapeutic protocol, which may not be based on a unique drug but rather on a combination of active antivirals.

AUTHOR CONTRIBUTIONS

IS: drafting the manuscript and literature review. VG: drafting the manuscript and illustration. FB, RS-S, and AJ: critical revision, editing, and approval of the final version. All of the authors read and approved the final manuscript.

FUNDING

The authors received financial support from the FAPEMIG (Minas Gerais Research Foundation APQ-00587-14 – SICONV 793988/2013; APQ-02872-16 and APQ-03385-18) and from CAPES (Coordination for the Improvement of Higher Education – #1678329P). AJ received a productivity fellowship from the CNPq (National Counsel of Technological and Scientific Development – 311219/2019-5). The Brazilian funding agencies CNPq, CAPES, and FAPEMIG provide financial support to the National Institute of Science and Technology in Theranostics and Nanobiotechnology – INCT-Teranano (CNPq-465669/2014-0). RS-S received a fellowship from FAU/UFU and PrInt CAPES/UFU. AJ, RS-S, and FB are grateful to CAPES – Prevention and Combat of Outbreaks, Endemics, Epidemics and Pandemics - Finance Code #88881.506794/2020-01.

ACKNOWLEDGMENTS

We thank the OSF Preprints database (https://osf.io/preprints/) for publishing the pre-print version of this manuscript on May 01, 2020 (https://doi.org/10.31219/osf.io/ycjgq).

REFERENCES

Adachi, A., and Miura, T. (2014). Animal model studies on viral infections. *Front. Microbiol.* 5:672. doi: 10.3389/fmicb.2014.00672

Adedeji, A. O., Severson, W., Jonsson, C., Singh, K., Weiss, S. R., and Sarafianos, S. G. (2013). Novel inhibitors of severe acute respiratory syndrome coronavirus entry that act by three distinct mechanisms. *J. Virol.* 87, 8017–8028. doi: 10.1128/JVI.00998-13

Agnihothram, S., Gopal, R., Yount, B. L., Donaldson, E. F., Menachery, V. D., Graham, R. L., et al. (2014). Evaluation of serologic and antigenic relationships between middle eastern respiratory syndrome coronavirus and other coronaviruses to develop vaccine platforms for the rapid response to emerging coronaviruses. *J. Infect. Dis.* 209, 995–1006. doi: 10.1093/infdis/jit609

Aguiar, A. C. C., Murce, E., Cortopassi, W. A., Pimentel, A. S., Almeida, M. M. F. S., Barros, D. C. S., et al. (2018). Chloroquine analogs as antimalarial candidates with potent in vitro and in vivo activity. *Int. J. Parasitol. Drugs Drug Resist.* 8, 459–464. doi: 10.1016/j.ijpddr.2018.10.002

Ahmed, S. F., Quadeer, A. A., and McKay, M. R. (2020). Preliminary identification of potential vaccine targets for the COVID-19 coronavirus (SARS-CoV-2) based on SARS-CoV immunological studies. *Viruses* 12:254. doi: 10.3390/v12030254

Åkerström, S., Mirazimi, A., and Tan, Y.-J. (2007). Inhibition of SARS-CoV replication cycle by small interference RNAs silencing specific SARS proteins, 7a/7b, 3a/3b and S. *Antiviral Res.* 73, 219–227. doi: 10.1016/j.antiviral.2006.10.008

Alattar, R., Ibrahim, T. B. H., Shaar, S. H., Abdalla, S., Shukri, K., Daghfal, J. N., et al. (2020). Tocilizumab for the treatment of severe coronavirus disease 2019. *J. Med. Virol.* Online ahead of print. doi: 10.1002/jmv.25964

Al-Bari, M. A. A. (2017). Targeting endosomal acidification by chloroquine analogs as a promising strategy for the treatment of emerging viral diseases. *Pharmacol. Res. Perspect.* 5, 1–13. doi: 10.1002/prp2.293

Al-Tawfiq, J. A., Momattin, H., Dib, J., and Memish, Z. A. (2014). Ribavirin and interferon therapy in patients infected with the Middle East respiratory syndrome coronavirus: an observational study. *Int. J. Infect. Dis.* 20, 42–46. doi: 10.1016/j.ijid.2013.12.003

Anand, K., Ziebuhr, J., Wadhwani, P., Mesters, J. R., and Hilgenfeld, R. (2003). Coronavirus main proteinase (3CLpro) structure: basis for design of anti-SARS drugs. *Science* 300, 1763–1767. doi: 10.1126/science.1085658

Andersen, K. G., Rambaut, A., Lipkin, W. I., Holmes, E. C., and Garry, R. F. (2020). The proximal origin of SARS-CoV-2. *Nat. Med.* 26, 450–452. doi: 10.1038/s41591-020-0820-9

Arya, R., Das, A., Prashar, V., and Kumar, M. (2020). Potential inhibitors against papain-like protease of novel coronavirus (SARS-CoV-2) from FDA approved drugs. *ChemRxiv [Preprint]* doi: 10.26434/chemrxiv.11860011.v2.

Axtell, R. C., de Jong, B. A., Boniface, K., van der Voort, L. F., Bhat, R., De Sarno, P., et al. (2010). T helper type 1 and 17 cells determine efficacy of interferon-β in multiple sclerosis and experimental encephalomyelitis. *Nat. Med.* 16, 406–412. doi: 10.1038/nm.2110

Balasubramaniam, M., and Reis, R. J. S. (2020). Computational target-based drug repurposing of elbasvir, an antiviral drug predicted to bind multiple SARS-CoV-2 proteins. *ChemRxiv [Preprint]* doi: 10.26434/chemrxiv.12084822

Balzarini, J., Keyaerts, E., Vijgen, L., Egberink, H., De Clercq, E., Van Ranst, M., et al. (2006). Inhibition of feline (FIPV) and human (SARS) coronavirus by semisynthetic derivatives of glycopeptide antibiotics. *Antiviral Res.* 72, 20–33. doi: 10.1016/j.antiviral.2006.03.005

Barnard, D. L., Day, C. W., Bailey, K., Heiner, M., Montgomery, R., Lauridsen, L., et al. (2006). Enhancement of the infectivity of SARS-CoV in BALB/c mice by IMP dehydrogenase inhibitors, including ribavirin. *Antiviral Res.* 71, 53–63. doi: 10.1016/j.antiviral.2006.03.001

Barnard, D. L., Hubbard, V. D., Burton, J., Smee, D. F., Morrey, J. D., Otto, M. J., et al. (2004). Inhibition of severe acute respiratory syndrome-associated coronavirus (SARSCoV) by calpain inhibitors and beta-D-N4-hydroxycytidine. *Antivir. Chem. Chemother.* 15, 15–22. doi: 10.1177/095632020401500102

Bayot, M. L., and King, K. C. (2020). *Biohazard Levels* Treasure Island, FL: StatPearls Publishing.

Bellingan, G., Maksimow, M., Howell, D. C., Stotz, M., Beale, R., Beatty, M., et al. (2014). The effect of intravenous interferon-beta-1a (FP-1201) on lung CD73 expression and on acute respiratory distress syndrome mortality: an open-label study. *Lancet Respir. Med.* 2, 98–107. doi: 10.1016/S2213-2600(13)70259-5

Belouzard, S., Chu, V. C., and Whittaker, G. R. (2009). Activation of the SARS coronavirus spike protein via sequential proteolytic cleavage at two distinct sites. *Proc. Natl. Acad. Sci. U.S.A.* 106, 5871–5876. doi: 10.1073/pnas.0809524106

Belouzard, S., Millet, J. K., Licitra, B. N., and Whittaker, G. R. (2012). Mechanisms of coronavirus cell entry mediated by the viral spike protein. *Viruses* 4, 1011–1033. doi: 10.3390/v4061011

Caly, L., Druce, J. D., Catton, M. G., Jans, D. A., and Wagstaff, K. M. (2020). The FDA-approved drug ivermectin inhibits the replication of SARS-CoV-2 in vitro. *Antiviral Res.* 178:104787. doi: 10.1016/j.antiviral.2020.104787

Cameron, M. C., Word, A. P., and Dominguez, A. (2014). Hydroxychloroquine-induced fatal toxic epidermal necrolysis complicated by angioinvasive rhizopus. *Dermatol. Online J.* 20:13030/qt1q90q0h5.

Campbell, W. C. (2012). *Ivermectin and Abamectin*. Berlin: Springer Science & Business Media.

Cao, H., and Shi, Y. (2020). Convalescent plasma: possible therapy for novel coronavirus disease 2019. *Transfusion* 60, 1078–1083. doi: 10.1111/trf.15797

Cao, Y., Li, L., Feng, Z., Wan, S., Huang, P., Sun, X., et al. (2020a). Comparative genetic analysis of the novel coronavirus (2019-nCoV/SARS-CoV-2) receptor ACE2 in different populations. *Cell Discov.* 6:11. doi: 10.1038/s41421-020-0147-1

Cao, B., Wang, Y., Wen, D., Liu, W., Wang, J., Fan, G., et al. (2020b). A trial of lopinavir–ritonavir in adults hospitalized with severe covid-19. *N. Engl. J. Med.* 382, 1787–1799. doi: 10.1056/NEJMoa2001282

CDC (2020a). *Coronavirus | Human Coronavirus Types | CDC*. Available online at: https://www.cdc.gov/coronavirus/types.html (accessed March 31, 2020)

CDC (2020b). *Information for Laboratories about Coronavirus (COVID-19)*. Available online at: https://www.cdc.gov/coronavirus/2019-ncov/lab/lab-biosafety-guidelines.html (accessed June 12, 2020)

Ceccarelli, M., Berretta, M., Rullo, E. V., Nunnari, G., and Cacopardo, B. (2020). Differences and similarities between severe acute respiratory syndrome (SARS)-CoronaVirus (CoV) and SARS-CoV-2. Would a rose by another name smell as sweet? *Eur. Rev. Med. Pharmacol. Sci.* 24, 2781–2783.

Chan, J. F. W., Chan, K.-H., Kao, R. Y. T., To, K. K. W., Zheng, B.-J., Li, C. P. Y., et al. (2013). Broad-spectrum antivirals for the emerging middle east respiratory syndrome coronavirus. *J. Infect.* 67, 606–616. doi: 10.1016/j.jinf.2013.09.029

Chan, J. F.-W., Yao, Y., Yeung, M.-L., Deng, W., Bao, L., Jia, L., et al. (2015). Treatment with lopinavir/ritonavir or interferon-β1b improves outcome of MERS-CoV infection in a nonhuman primate model of common marmoset. *J. Infect. Dis.* 212, 1904–1913. doi: 10.1093/infdis/jiv392

Chen, Y., Liu, Q., and Guo, D. (2020). Emerging coronaviruses: genome structure, replication, and pathogenesis. *J. Med. Virol.* 92, 418–423. doi: 10.1002/jmv.25681

Chen, J., Liu, D., Liu, L., Liu, P., Xu, Q., Xia, L., et al. (2020). [A pilot study of hydroxychloroquine in treatment of patients with moderate COVID-19]. *Zhejiang Xue Xue Bao Yi Xue Ban J. Zhejiang Univ. Med. Sci.* 49, 215–219.

Chen, L., Xiong, J., Bao, L., and Shi, Y. (2020). Convalescent plasma as a potential therapy for COVID-19. *Lancet Infect. Dis.* 20, 398–400. doi: 10.1016/S1473-3099(20)30141-9

Chen, L.-R., Wang, Y.-C., Lin, Y. W., Chou, S.-Y., Chen, S.-F., Liu, L. T., et al. (2005). Synthesis and evaluation of isatin derivatives as effective SARS coronavirus 3CL protease inhibitors. *Bioorg. Med. Chem. Lett.* 15, 3058–3062. doi: 10.1016/j.bmcl.2005.04.027

Chen, W.-H., Strych, U., Hotez, P. J., and Bottazzi, M. E. (2020). The SARS-CoV-2 Vaccine Pipeline: an Overview. *Curr. Trop. Med. Rep.* 3, 1–4. doi: 10.1007/s40475-020-00201-6

Chen, Z., Hu, J., Zhang, Z., Jiang, S., Han, S., Yan, D., et al. (2020). Efficacy of hydroxychloroquine in patients with COVID-19: results of a randomized clinical trial. *medRxiv [Preprint]* doi: 10.1101/2020.03.22.20040758

Cheng, Y., Wong, R., Soo, Y. O. Y., Wong, W. S., Lee, C. K., Ng, M. H. L., et al. (2005). Use of convalescent plasma therapy in SARS patients in Hong Kong. *Eur. J. Clin. Microbiol. Infect. Dis.* 24, 44–46. doi: 10.1007/s10096-004-1271-9

Chihrin, S., and Loutfy, M. R. (2005). Overview of antiviral and anti-inflammatory treatment for severe acute respi ratory syndrome. *Expert Rev. Anti Infect. Ther.* 3, 251–262. doi: 10.1586/14787210.3.2.251

Chorin, E., Dai, M., Shulman, E., Wadhwani, L., Cohen, R. B., Barbhaiya, C., et al. (2020). The QT Interval in Patients with SARS-CoV-2 Infection Treated with Hydroxychloroquine/Azithromycin. *medRxiv [Preprint]* doi: 10.1101/2020.04.02.20047050

Chousterman, B. G., Swirski, F. K., and Weber, G. F. (2017). Cytokine storm and sepsis disease pathogenesis. *Semin. Immunopathol.* 39, 517–528. doi: 10.1007/s00281-017-0639-8

Chu, C. M., Cheng, V. C. C., Hung, I. F. N., Wong, M. M. L., Chan, K. H., Chan, K. S., et al. (2004). Role of lopinavir/ritonavir in the treatment of SARS: initial virological and clinical findings. *Thorax* 59, 252–256. doi: 10.1136/thorax.2003.012658

Chu, H., Chan, J. F.-W., Yuen, T. T.-T., Shuai, H., Yuan, S., Wang, Y., et al. (2020). Comparative tropism, replication kinetics, and cell damage profiling of SARS-CoV-2 and SARS-CoV with implications for clinical manifestations, transmissibility, and laboratory studies of COVID-19: an observational study. *Lancet Microbe* 1, e14–e23. doi: 10.1016/S2666-5247(20)30004-5

Cinatl, J., Michaelis, M., Hoever, G., Preiser, W., and Doerr, H. W. (2005). Development of antiviral therapy for severe acute respiratory syndrome. *Antiviral Res.* 66, 81–97. doi: 10.1016/j.antiviral.2005.03.002

Cinatl, J., Morgenstern, B., Bauer, G., Chandra, P., Rabenau, H., and Doerr, H. W. (2003). Glycyrrhizin, an active component of liquorice roots, and replication of SARS-associated coronavirus. *Lancet* 361, 2045–2046. doi: 10.1016/S0140-6736(03)13615-X

Clark, L. T., Watkins, L., Piña, I. L., Elmer, M., Akinboboye, O., Gorham, M., et al. (2019). Increasing diversity in clinical trials: overcoming critical barriers. *Curr. Probl. Cardiol.* 44, 148–172. doi: 10.1016/j.cpcardiol.2018.11.002

Cohn, L. A. (1991). The influence of corticosteroids on host defense mechanisms. *J. Vet. Intern. Med.* 5, 95–104. doi: 10.1111/j.1939-1676.1991.tb00939.x

Coleman, C. M., and Frieman, M. B. (2014). Coronaviruses: important emerging human pathogens. *J. Virol.* 88, 5209–5212. doi: 10.1128/JVI.03488-13

Cui, J., Li, F., and Shi, Z.-L. (2019). Origin and evolution of pathogenic coronaviruses. *Nat. Rev. Microbiol.* 17, 181–192. doi: 10.1038/s41579-018-0118-9

Cvetkovic, R. S., and Goa, K. L. (2003). Lopinavir/Ritonavir. *Drugs* 63, 769–802. doi: 10.2165/00003495-200363080-00004

Dai, C., Ma, Y., Zhao, Z., Zhao, R., Wang, Q., Wu, Y., et al. (2008). Mucroporin, the first cationic host defense peptide from the venom of lychas mucronatus. *Antimicrob. Agents Chemother.* 52, 3967–3972. doi: 10.1128/AAC.00542-08

D'Amato, G., Stanziola, A., Sanduzzi, A., Liccardi, G., Salzillo, A., Vitale, C., et al. (2014). Treating severe allergic asthma with anti-IgE monoclonal antibody (omalizumab): a review. *Multidiscip. Respir. Med.* 9:23. doi: 10.1186/2049-6958-9-23

Davis, T. L., Walker, J. R., Campagna-Slater, V., Finerty, P. J., Paramanathan, R., Bernstein, G., et al. (2010). Structural and biochemical characterization of the human cyclophilin family of peptidyl-prolyl isomerases. *PLoS Biol.* 8:e1000439. doi: 10.1371/journal.pbio.1000439

Day, C. W., Baric, R., Cai, S. X., Frieman, M., Kumaki, Y., Morrey, J. D., et al. (2009). A new mouse-adapted strain of SARS-CoV as a lethal model for evaluating antiviral agents in vitro and in vivo. *Virology* 395, 210–222. doi: 10.1016/j.virol.2009.09.023

de Wilde, A. H., Falzarano, D., Zevenhoven-Dobbe, J. C., Beugeling, C., Fett, C., Martellaro, C., et al. (2017). Alisporivir inhibits MERS- and SARS-coronavirus replication in cell culture, but not SARS-coronavirus infection in a mouse model. *Virus Res.* 228, 7–13. doi: 10.1016/j.virusres.2016.11.011

de Wilde, A. H., Jochmans, D., Posthuma, C. C., Zevenhoven-Dobbe, J. C., Nieuwkoop, S., van, et al. (2014). Screening of an FDA-approved compound library identifies four small-molecule inhibitors of middle east respiratory syndrome coronavirus replication in cell culture. *Antimicrob. Agents Chemother.* 58, 4875–4884. doi: 10.1128/AAC.03011-14

de Wilde, A. H., Raj, V. S., Oudshoorn, D., Bestebroer, T. M., van Nieuwkoop, S., Limpens, R. W. A. L., et al. (2013). MERS-coronavirus replication induces severe in vitro cytopathology and is strongly inhibited by cyclosporin A or interferon-α treatment. *J. Gen. Virol.* 94, 1749–1760. doi: 10.1099/vir.0.052910-0

de Wilde, A. H., Zevenhoven-Dobbe, J. C., van der Meer, Y., Thiel, V., Narayanan, K., Makino, S., et al. (2011). Cyclosporin A inhibits the replication of diverse coronaviruses. *J. Gen. Virol.* 92, 2542–2548. doi: 10.1099/vir.0.034983-0

De Wit, E., Feldmann, F., Cronin, J., Jordan, R., Okumura, A., Thomas, T., et al. (2020). Prophylactic and therapeutic remdesivir (GS-5734) treatment in the rhesus macaque model of MERS-CoV infection. *Proc. Natl. Acad. Sci. U.S.A.* 117, 6771–6776. doi: 10.1073/pnas.1922083117

Dhama, K., Sharun, K., Tiwari, R., Dadar, M., Malik, Y. S., Singh, K. P., et al. (2020). COVID-19, an emerging coronavirus infection: advances and prospects in designing and developing vaccines, immunotherapeutics, and therapeutics. *Hum. Vaccines Immunother.* 2020, 1–7. doi: 10.1080/21645515.2020.1735227

Duan, K., Liu, B., Li, C., Zhang, H., Yu, T., Qu, J., et al. (2020). Effectiveness of convalescent plasma therapy in severe COVID-19 patients. *Proc. Natl. Acad. Sci. U.S.A.* 117, 9490–9496. doi: 10.1073/pnas.2004168117

El-Solia, A., Al-Otaibi, K., and Ai-Hwiesh, A. K. (2018). Hydroxychloroquine-induced hypoglycaemia in non-diabetic renal patient on peritoneal dialysis. *BMJ Case Rep.* 2018:bcr2017223639. doi: 10.1136/bcr-2017-223639

Falzarano, D., de Wit, E., Martellaro, C., Callison, J., Munster, V. J., and Feldmann, H. (2013a). Inhibition of novel β coronavirus replication by a combination of interferon-α2b and ribavirin. *Sci. Rep.* 3:1686. doi: 10.1038/srep01686

Falzarano, D., de Wit, E., Rasmussen, A. L., Feldmann, F., Okumura, A., Scott, D. P., et al. (2013b). Treatment with interferon-α2b and ribavirin improves outcome in MERS-CoV–infected rhesus macaques. *Nat. Med.* 19, 1313–1317. doi: 10.1038/nm.3362

FDA (2020). *FDA Cautions Against use of Hydroxychloroquine or Chloroquine for COVID-19 Outside of the Hospital Setting or a Clinical trial due to Risk of Heart Rhythm Problems*. Available online at: https://www.fda.gov/drugs/drug-safety-and-availability/fda-cautions-against-use-hydroxychloroquine-or-chloroquine-covid-19-outside-hospital-setting-or (accessed July 5, 2020)

Fehr, A. R., and Perlman, S. (2015). Coronaviruses: an overview of their replication and pathogenesis. *Methods Mol. Biol. Clifton NJ* 1282, 1–23. doi: 10.1007/978-1-4939-2438-7_1

Ferron, F., Subissi, L., De Morais, A. T. S., Le, N. T. T., Sevajol, M., Gluais, L., et al. (2017). Structural and molecular basis of mismatch correction and ribavirin excision from coronavirus RNA. *Proc. Natl. Acad. Sci. U S A.* 115, E162–E171. doi: 10.1073/pnas.1718806115

Gan, Y.-R., Huang, H., Huang, Y.-D., Rao, C.-M., Zhao, Y., Liu, J.-S., et al. (2006). Synthesis and activity of an octapeptide inhibitor designed for SARS coronavirus main proteinase. *Peptides* 27, 622–625. doi: 10.1016/j.peptides.2005.09.006

Ganeshpurkar, A., Gutti, G., and Singh, S. K. (2019). "Chapter 1 - RNA-dependent RNA polymerases and their emerging roles in antiviral therapy," in *Viral Polymerases*, ed. S. P. Gupta (Cambridge, MA: Academic Press), 1–42. doi: 10.1016/B978-0-12-815422-9.00001-2

Gao, J., Tian, Z., and Yang, X. (2020). Breakthrough: chloroquine phosphate has shown apparent efficacy in treatment of COVID-19 associated pneumonia in clinical studies. *Biosci. Trends* 14, 72–73. doi: 10.5582/BST.2020.01047

Garraud, O. (2017). Use of convalescent plasma in Ebola virus infection. *Transfus. Apher. Sci.* 56, 31–34. doi: 10.1016/j.transci.2016.12.014

Gautret, P., Lagier, J.-C., Parola, P., Hoang, V. T., Meddeb, L., Mailhe, M., et al. (2020). Hydroxychloroquine and azithromycin as a treatment of COVID-19: results of an open-label non-randomized clinical trial. *Int. J. Antimicrob. Agents* 20:105949. doi: 10.1016/j.ijantimicag.2020.105949

Giles, A. J., Hutchinson, M.-K. N. D., Sonnemann, H. M., Jung, J., Fecci, P. E., Ratnam, N. M., et al. (2018). Dexamethasone-induced immunosuppression: mechanisms and implications for immunotherapy. *J. Immunother. Cancer* 6:51. doi: 10.1186/s40425-018-0371-5

Girijala, R. L., Siddiqi, I., Kwak, Y., Wright, D., Patel, D. B., and Goldberg, L. H. (2019). Pustular DRESS syndrome secondary to hydroxychloroquine with EBV reactivation. *J. Drugs Dermatol. JDD* 18, 207–209.

Gorbalenya, A. E., Baker, S. C., Baric, R. S., de Groot, R. J., Drosten, C., Gulyaeva, A. A., et al. (2020a). Severe acute respiratory syndrome-related coronavirus: the species and its viruses – a statement of the Coronavirus study group. *BioRxiv [Preprint]* doi: 10.1101/2020.02.07.937862

Gorbalenya, A. E., Baker, S. C., Baric, R. S., de Groot, R. J., Drosten, C., Gulyaeva, A. A., et al. (2020b). The species Severe acute respiratory syndrome-related coronavirus: classifying 2019-nCoV and naming it SARS-CoV-2. *Nat. Microbiol.* 5, 536–544. doi: 10.1038/s41564-020-0695-z

Götz, V., Magar, L., Dornfeld, D., Giese, S., Pohlmann, A., Höper, D., et al. (2016). Influenza A viruses escape from MxA restriction at the expense of efficient nuclear vRNP import. *Sci. Rep.* 6:23138. doi: 10.1038/srep23138

Graham, R. L., Donaldson, E. F., and Baric, R. S. (2013). A decade after SARS: strategies for controlling emerging coronaviruses. *Nat. Rev. Microbiol.* 11, 836–848. doi: 10.1038/nrmicro3143

Green, M. C., Murray, J. L., and Hortobagyi, G. N. (2000). Monoclonal antibody therapy for solid tumors. *Cancer Treat. Rev.* 26, 269–286. doi: 10.1053/ctrv.2000.0176

Grupp, S. A., Kalos, M., Barrett, D., Aplenc, R., Porter, D. L., Rheingold, S. R., et al. (2013). Chimeric antigen receptor–modified T cells for acute lymphoid leukemia. *N. Engl. J. Med.* 368, 1509–1518. doi: 10.1056/NEJMoa1215134

Guan, B.-J., Su, Y.-P., Wu, H.-Y., and Brian, D. A. (2012). Genetic evidence of a long-range RNA-RNA interaction between the genomic 5' untranslated region and the nonstructural protein 1 coding region in murine and bovine Coronaviruses. *J. Virol.* 86, 4631–4643. doi: 10.1128/JVI.06265-11

Guan, W., Ni, Z., Hu, Y., Liang, W., Ou, C., He, J., et al. (2020). Clinical characteristics of Coronavirus disease 2019 in China. *N. Engl. J. Med.* 382, 1708–1720. doi: 10.1056/NEJMoa2002032

Haga, S., Nagata, N., Okamura, T., Yamamoto, N., Sata, T., Yamamoto, N., et al. (2010). TACE antagonists blocking ACE2 shedding caused by the spike protein of SARS-CoV are candidate antiviral compounds. *Antiviral Res.* 85, 551–555. doi: 10.1016/j.antiviral.2009.12.001

Haga, S., Yamamoto, N., Nakai-Murakami, C., Osawa, Y., Tokunaga, K., Sata, T., et al. (2008). Modulation of TNF- -converting enzyme by the spike protein of SARS-CoV and ACE2 induces TNF- production and facilitates viral entry. *Proc. Natl. Acad. Sci. U.S.A.* 105, 7809–7814. doi: 10.1073/pnas.0711241105

Han, D. P., Lohani, M., and Cho, M. W. (2007). Specific asparagine-linked glycosylation sites are critical for DC-SIGN- and L-SIGN-mediated severe acute respiratory syndrome Coronavirus entry. *J. Virol.* 81, 12029–12039. doi: 10.1128/JVI.00315-07

Hart, B. J., Dyall, J., Postnikova, E., Zhou, H., Kindrachuk, J., Johnson, R. F., et al. (2014). Interferon-β and mycophenolic acid are potent inhibitors of middle east respiratory syndrome coronavirus in cell-based assays. *J. Gen. Virol.* 95, 571–577. doi: 10.1099/vir.0.061911-0

Haynes, L. M., Caidi, H., Radu, G. U., Miao, C., Harcourt, J. L., Tripp, R. A., et al. (2009). Therapeutic monoclonal antibody treatment targeting respiratory syncytial virus (RSV) G protein mediates viral clearance and reduces the pathogenesis of RSV infection in BALB/c mice. *J. Infect. Dis.* 200, 439–447. doi: 10.1086/600108

Hensley, L. E., Fritz, E. A., Jahrling, P. B., Karp, C., Huggins, J. W., and Geisbert, T. W. (2004). Interferon-β 1a and SARS Coronavirus Replication. *Emerg. Infect. Dis.* 10, 317–319. doi: 10.3201/eid1002.030482

Hess, A. S., and Abd-Elsayed, A. (2019). "Observational studies: uses and limitations," in *Pain: A Review Guide*, ed. A. Abd-Elsayed (Cham: Springer International Publishing), 123–125. doi: 10.1007/978-3-319-99124-5_31

Ho, T.-Y., Wu, S.-L., Chen, J.-C., Li, C.-C., and Hsiang, C.-Y. (2007). Emodin blocks the SARS coronavirus spike protein and angiotensin-converting enzyme 2 interaction. *Antiviral Res.* 74, 92–101. doi: 10.1016/j.antiviral.2006.04.014

Hoever, G., Baltina, L., Michaelis, M., Kondratenko, R., Baltina, L., Tolstikov, G. A., et al. (2005). Antiviral activity of glycyrrhizic acid derivatives against SARS-Coronavirus. *J. Med. Chem.* 48, 1256–1259. doi: 10.1021/jm0493008

Hoffmann, M., Kleine-Weber, H., Schroeder, S., Krüger, N., Herrler, T., Erichsen, S., et al. (2020). SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor. *Cell* 181, 271.e8–280.e8. doi: 10.1016/j.cell.2020.02.052

Holshue, M. L., DeBolt, C., Lindquist, S., Lofy, K. H., Wiesman, J., Bruce, H., et al. (2020). First case of 2019 novel coronavirus in the united states. *N. Engl. J. Med.* 382, 929–936. doi: 10.1056/NEJMoa2001191

Horby, P., Lim, W. S., Emberson, J., Mafham, M., Bell, J., Linsell, L., et al. (2020). Effect of dexamethasone in hospitalized patients with COVID-19: preliminary report. *medRxiv [Preprint]* doi: 10.1101/2020.06.22.20137273

Hsu, L. Y., Chia, P. Y., and Lim, J. F. (2020). The novel Coronavirus (SARS-CoV-2) epidemic. *Ann. Acad. Med. Singapore* 49, 1–3.

Huang, I.-C., Bosch, B. J., Li, F., Li, W., Lee, K. H., Ghiran, S., et al. (2006). SARS coronavirus, but not human coronavirus NL63, utilizes cathepsin L to infect ACE2-expressing cells. *J. Biol. Chem.* 281, 3198–3203. doi: 10.1074/jbc.M508381200

Huentelman, M. J., Zubcevic, J., Hernández Prada, J. A., Xiao, X., Dimitrov, D. S., Raizada, M. K., et al. (2004a). Structure-based discovery of a novel angiotensin-converting enzyme 2 inhibitor. *Hypertension* 44, 903–906. doi: 10.1161/01.HYP.0000146120.29648.36

Huentelman, M. J., Zubcevic, J., Katovich, M. J., and Raizada, M. K. (2004b). Cloning and characterization of a secreted form of angiotensin-converting enzyme 2. *Regul. Pept.* 122, 61–67. doi: 10.1016/j.regpep.2004.05.003

Hung, I. F.-N., Lung, K.-C., Tso, E. Y.-K., Liu, R., Chung, T. W.-H., Chu, M.-Y., et al. (2020). Triple combination of interferon beta-1b, lopinavir-ritonavir, and ribavirin in the treatment of patients admitted to hospital with COVID-19: an open-label, randomised, phase 2 trial. *Lancet Lond. Engl.* 395, 1695–1704. doi: 10.1016/S0140-6736(20)31042-4

Huynh, J., Li, S., Yount, B., Smith, A., Sturges, L., Olsen, J. C., et al. (2012). Evidence supporting a zoonotic origin of human Coronavirus strain NL63. *J. Virol.* 86, 12816–12825. doi: 10.1128/JVI.00906-12

Jacobson, J. M., Bosinger, S. E., Kang, M., Belaunzaran-Zamudio, P., Matining, R. M., Wilson, C. C., et al. (2016). The effect of chloroquine on immune activation and interferon signatures associated with HIV-1. *AIDS Res. Hum. Retroviruses* 32, 636–647. doi: 10.1089/aid.2015.0336

Jasenosky, L. D., Cadena, C., Mire, C. E., Borisevich, V., Haridas, V., Ranjbar, S., et al. (2019). The FDA-approved oral drug nitazoxanide amplifies host antiviral responses and inhibits ebola virus. *iScience* 19, 1279–1290. doi: 10.1016/j.isci.2019.07.003

Jeffers, S. A., Tusell, S. M., Gillim-Ross, L., Hemmila, E. M., Achenbach, J. E., Babcock, G. J., et al. (2004). CD209L (L-SIGN) is a receptor for severe acute respiratory syndrome coronavirus. *Proc. Natl. Acad. Sci. U.S.A.* 101, 15748–15753. doi: 10.1073/pnas.0403812101

Jeon, S., Ko, M., Lee, J., Choi, I., Byun, S. Y., Park, S., et al. (2020). Identification of antiviral drug candidates against SARS-CoV-2 from FDA-approved drugs. *Antimicrob. Agents Chemother.* 64, e00819–e00820. doi: 10.1128/AAC.00819-20

Juurlink, D. N. (2020). Safety considerations with chloroquine, hydroxychloroquine and azithromycin in the management of SARS-CoV-2 infection. *CMAJ* 192, E450–E453. doi: 10.1503/cmaj.200528

Kaeppler, U., Stiefl, N., Schiller, M., Vicik, R., Breuning, A., Schmitz, W., et al. (2005). A new lead for nonpeptidic active-site-directed inhibitors of the severe acute respiratory syndrome coronavirus main protease discovered by a combination of screening and docking methods. *J. Med. Chem.* 48, 6832–6842. doi: 10.1021/jm0501782

Kahn, J. S., and McIntosh, K. (2005). History and recent advances in coronavirus discovery. *Pediatr. Infect Dis. J.* 24(11 Suppl), S223-S227.

Karamitros, T., Papadopoulou, G., Bousali, M., Mexias, A., Tsiodras, S., and Mentis, A. (2020). SARS-CoV-2 exhibits intra-host genomic plasticity and low-frequency polymorphic quasispecies. *bioRxiv [Preprint]* doi: 10.1101/2020.03.27.009480

Katz, M. (1977). Anthelmintics. *Drugs* 13, 124–136. doi: 10.2165/00003495-197713020-00002

Kernan, W. N., Viscoli, C. M., Makuch, R. W., Brass, L. M., and Horwitz, R. I. (1999). Stratified randomization for clinical trials. *J. Clin. Epidemiol.* 52, 19–26. doi: 10.1016/S0895-4356(98)00138-3

Kesel, A. J. (2003). A system of protein target sequences for anti-RNA-viral chemotherapy by a vitamin B6-Derived zinc-Chelating trioxa-adamantane-triol. *Bioorg. Med. Chem.* 11, 4599–4613. doi: 10.1016/S0968-0896(03)00500-5

Khamitov, R., Loginova, S., Borisevich, S., Maksimov, V., and Shuster, A. (2008). *Antiviral Activity of Arbidol and its Derivatives against the Pathogen of severe Acute Respiratory Syndrome in the Cell Cultures*. 9–13. Available online at: https://europepmc.org/article/med/18756809 (accessed June 10, 2020).

Kim, D., Lee, J., Yang, J., Kim, J. W., Kim, V. N., and Chang, H. (2020). The architecture of SARS-CoV-2 transcriptome. *Cell* 181, 914.e10–921.e10. doi: 10.1016/j.cell.2020.04.011

Kim, Y., Lovell, S., Tiew, K.-C., Mandadapu, S. R., Alliston, K. R., Battaile, K. P., et al. (2012). Broad-spectrum antivirals against 3C or 3C-like proteases of picornaviruses, noroviruses, and Coronaviruses. *J. Virol.* 86, 11754–11762. doi: 10.1128/JVI.01348-12

Kim, Y., Mandadapu, S. R., Groutas, W. C., and Chang, K.-O. (2013). Potent inhibition of feline coronaviruses with peptidyl compounds targeting coronavirus 3C-like protease. *Antiviral Res.* 97, 161–168. doi: 10.1016/j.antiviral.2012.11.005

Kim, U. J., Won, E.-J., Kee, S.-J., Jung, S.-I., and Jang, H.-C. (2015). Combination therapy with lopinavir/ritonavir, ribavirin and interferon-α for Middle East respiratory syndrome: a case report. *Antivir. Ther.* 21, 455–459. doi: 10.3851/IMP3002

Kipar, A., Meli, M. L., Baptiste, K. E., Bowker, L. J., and Lutz, H. (2010). Sites of feline coronavirus persistence in healthy cats. *J. Gen. Virol.* 91, 1698–1707. doi: 10.1099/vir.0.020214-0

Kirchheiner, J., Keulen, J.-T. H. A., Bauer, S., Roots, I., and Brockmöller, J. (2008). Effects of the CYP2D6 gene duplication on the pharmacokinetics and pharmacodynamics of tramadol. *J. Clin. Psychopharmacol.* 28, 78–83. doi: 10.1097/JCP.0b013e318160f827

Ko, J.-H., Seok, H., Cho, S. Y., Ha, Y. E., Baek, J. Y., Kim, S. H., et al. (2018). Challenges of convalescent plasma infusion therapy in Middle East respiratory Coronavirus infection: a single centre experience. *Antivir. Ther.* 23, 617–622. doi: 10.3851/IMP3243

Kobayashi, Y., Watanabe, S., Konishi, M., Yokoi, M., Kakehashi, R., Kaito, M., et al. (1993). Quantitation and typing of serum hepatitis C virus RNA in patients with chronic hepatitis C treated with interferon-β. *Hepatology* 18, 1319–1325. doi: 10.1002/hep.1840180606

Lai, C.-C., Shih, T.-P., Ko, W.-C., Tang, H.-J., and Hsueh, P.-R. (2020). Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and coronavirus disease-2019 (COVID-19): the epidemic and the challenges. *Int. J. Antimicrob. Agents* 55:105924. doi: 10.1016/j.ijantimicag.2020.105924

Lai, M. M., and Stohlman, S. A. (1981). Comparative analysis of RNA genomes of mouse hepatitis viruses. *J. Virol.* 38, 661–670. doi: 10.1128/jvi.38.2.661-670.1981

Laing, R., Gillan, V., and Devaney, E. (2017). Ivermectin – old drug. New Tricks? *Trends Parasitol.* 33, 463–472. doi: 10.1016/j.pt.2017.02.004

Laplante, J., and St George, K. (2014). Antiviral resistance in influenza viruses: laboratory testing. *Clin. Lab. Med.* 34, 387–408. doi: 10.1016/j.cll.2014.02.010

Lee, J. Y., Vinayagamoorthy, N., Han, K., Kwok, S. K., Ju, J. H., Park, K. S., et al. (2016). Association of polymorphisms of cytochrome P450 2D6 with blood

hydroxychloroquine levels in patients with systemic lupus erythematosus. *Arthritis Rheumatol.* 68, 184–190. doi: 10.1002/art.39402

Lee, T.-W., Cherney, M. M., Huitema, C., Liu, J., James, K. E., Powers, J. C., et al. (2005). Crystal structures of the main peptidase from the SARS Coronavirus Inhibited by a substrate-like aza-peptide epoxide. *J. Mol. Biol.* 353, 1137–1151. doi: 10.1016/j.jmb.2005.09.004

Lengauer, T., and Sing, T. (2006). Bioinformatics-assisted anti-HIV therapy. *Nat. Rev. Microbiol.* 4, 790–797. doi: 10.1038/nrmicro1477

Li, D. K., and Chung, R. T. (2019). Overview of direct-acting antiviral drugs and drug resistance of hepatitis C Virus. *Methods Mol. Biol. Clifton NJ* 1911, 3–32. doi: 10.1007/978-1-4939-8976-8_1

Li, K., Li, H., Bi, Z., Song, D., Zhang, F., Lei, D., et al. (2019). Significant inhibition of re-emerged and emerging swine enteric coronavirus in vitro using the multiple shRNA expression vector. *Antiviral Res.* 166, 11–18. doi: 10.1016/j.antiviral.2019.03.010

Li, Q., Zhao, Z., Zhou, D., Chen, Y., Hong, W., Cao, L., et al. (2011). Virucidal activity of a scorpion venom peptide variant mucroporin-M1 against measles, SARS-CoV and influenza H5N1 viruses. *Peptides* 32, 1518–1525. doi: 10.1016/j.peptides.2011.05.015

Li, X., Geng, M., Peng, Y., Meng, L., and Lu, S. (2020). Molecular immune pathogenesis and diagnosis of COVID-19. *J. Pharm. Anal.* 10, 102–108. doi: 10.1016/j.jpha.2020.03.001

Lian, N., Xie, H., Lin, S., Huang, J., Zhao, J., and Lin, Q. (2020). Umifenovir treatment is not associated with improved outcomes in patients with coronavirus disease 2019: a retrospective study. *Clin. Microbiol. Infect.* 26, 917–921. doi: 10.1016/j.cmi.2020.04.026

Lip, K.-M., Shen, S., Yang, X., Keng, C.-T., Zhang, A., Oh, H.-L. J., et al. (2006). Monoclonal antibodies targeting the HR2 domain and the region immediately upstream of the HR2 of the S protein neutralize in vitro infection of severe acute respiratory syndrome coronavirus. *J. Virol.* 80, 941–950. doi: 10.1128/JVI.80.2.941-950.2006

Lipinski, C. A., Lombardo, F., Dominy, B. W., and Feeney, P. J. (2001). Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv. Drug Deliv. Rev.* 46, 3–26. doi: 10.1016/s0169-409x(00)00129-0

Lipman, N. S., Jackson, L. R., Trudel, L. J., and Weis-Garcia, F. (2005). Monoclonal versus polyclonal antibodies: distinguishing characteristics, applications, and information resources. *ILAR J.* 46, 258–268. doi: 10.1093/ilar.46.3.258

Liu, J., Cao, R., Xu, M., Wang, X., Zhang, H., Hu, H., et al. (2020). Hydroxychloroquine, a less toxic derivative of chloroquine, is effective in inhibiting SARS-CoV-2 infection in vitro. *Cell Discov.* 6, 1–4. doi: 10.1038/s41421-020-0156-0

Lu, G., Wang, Q., and Gao, G. F. (2015). Bat-to-human: spike features determining 'host jump' of coronaviruses SARS-CoV, MERS-CoV, and beyond. *Trends Microbiol.* 23, 468–478. doi: 10.1016/j.tim.2015.06.003

Lusvarghi, S., and Bewley, C. A. (2016). Griffithsin: an antiviral lectin with outstanding therapeutic potential. *Viruses* 8:296. doi: 10.3390/v8100296

Ma, Y., Chan, C. Y., and He, M. L. (2007). RNA interference and antiviral therapy. *World J. Gastroenterol.* 13, 5169–5179. doi: 10.3748/wjg.v13.i39.5169

Madhugiri, R., Karl, N., Petersen, D., Lamkiewicz, K., Fricke, M., Wend, U., et al. (2018). Structural and functional conservation of cis-acting RNA elements in coronavirus 5'-terminal genome regions. *Virology* 517, 44–55. doi: 10.1016/j.virol.2017.11.025

Marasco, W. A., and Sui, J. (2007). The growth and potential of human antiviral monoclonal antibody therapeutics. *Nat. Biotechnol.* 25, 1421–1434. doi: 10.1038/nbt1363

Matsuyama, S., Nao, N., Shirato, K., Kawase, M., Saito, S., Takayama, I., et al. (2020). Enhanced isolation of SARS-CoV-2 by TMPRSS2-expressing cells. *Proc. Natl. Acad. Sci. U.S.A.* 117, 7001–7003. doi: 10.1073/pnas.2002589117

Mauthe, M., Orhon, I., Rocchi, C., Zhou, X., Luhr, M., Hijlkema, K.-J., et al. (2018). Chloroquine inhibits autophagic flux by decreasing autophagosome-lysosome fusion. *Autophagy* 14, 1435–1455. doi: 10.1080/15548627.2018.1474314

McDonagh, P., Sheehy, P. A., and Norris, J. M. (2011). In vitro inhibition of feline coronavirus replication by small interfering RNAs. *Vet. Microbiol.* 150, 220–229. doi: 10.1016/j.vetmic.2011.01.023

McDonagh, P., Sheehy, P. A., and Norris, J. M. (2015). Combination siRNA therapy against feline coronavirus can delay the emergence of antiviral resistance in vitro. *Vet. Microbiol.* 176, 10–18. doi: 10.1016/j.vetmic.2014.12.009

Mehta, P., McAuley, D. F., Brown, M., Sanchez, E., Tattersall, R. S., and Manson, J. J. (2020). COVID-19: consider cytokine storm syndromes and immunosuppression. *Lancet Lond. Engl.* 395, 1033–1034. doi: 10.1016/S0140-6736(20)30628-0

Millet, J. K., Séron, K., Labitt, R. N., Danneels, A., Palmer, K. E., Whittaker, G. R., et al. (2016). Middle East respiratory syndrome coronavirus infection is inhibited by griffithsin. *Antiviral Res.* 133, 1–8. doi: 10.1016/j.antiviral.2016.07.011

Mills, A. M., Nelson, M., Jayaweera, D., Ruxrungtham, K., Cassetti, I., Girard, P.-M., et al. (2009). Once-daily darunavir/ritonavir vs. lopinavir/ritonavir in treatment-naive, HIV-1-infected patients: 96-week analysis. *AIDS* 23, 1679–1688. doi: 10.1097/QAD.0b013e32832d7350

Mohan, D., Mohandas, E., and Rajat, R. (1981). Chloroquine psychosis: a chemical psychosis? *J. Natl. Med. Assoc.* 73, 1073–1076.

Molina, J. M., Delaugerre, C., Le Goff, J., Mela-Lima, B., Ponscarme, D., Goldwirt, L., et al. (2020). No evidence of rapid antiviral clearance or clinical benefit with the combination of hydroxychloroquine and azithromycin in patients with severe COVID-19 infection. *Med. Mal. Infect.* 50:384. doi: 10.1016/j.medmal.2020.03.006

Morra, M. E., Van Thanh, L., Kamel, M. G., Ghazy, A. A., Altibi, A. M. A., Dat, L. M., et al. (2018). Clinical outcomes of current medical approaches for Middle East respiratory syndrome: a systematic review and meta-analysis. *Rev. Med. Virol.* 28:e1977. doi: 10.1002/rmv.1977

Mullard, A. (2020). COVID-19 vaccine development pipeline gears up. *Lancet* 395, 1751–1752. doi: 10.1016/S0140-6736(20)31252-6

Murphy, F., and Middleton, M. (2012). "45 - Cytostatic and cytotoxic drugs," in *Side Effects of Drugs Annual A Worldwide Yearly Survey of New data in Adverse drug Reactions and Interactions*, ed. J. K. Aronson (Amsterdam: Elsevier), 731–747. doi: 10.1016/B978-0-444-59499-0.00045-3

Mzayek, F., Deng, H., Mather, F. J., Wasilevich, E. C., Liu, H., Hadi, C. M., et al. (2007). Randomized dose-ranging controlled trial of AQ-13, a candidate antimalarial, and chloroquine in healthy volunteers. *PLoS Clin. Trials* 2:e6. doi: 10.1371/journal.pctr.0020006

National Research Council (US) Committee on Applications of Toxicogenomic Technologies to Predictive Toxicology (2007). *Application to the Study of Mechanisms of Action*. Washington DC: National Academies Press.

Natoli, S., Oliveira, V., Calabresi, P., Maia, L. F., and Pisani, A. (2020). Does SARS-Cov-2 invade the brain? Translational lessons from animal models. *Eur. J. Neurol.* 22:10.1111/ene.14277. doi: 10.1111/ene.14277

O'Keefe, B. R., Giomarelli, B., Barnard, D. L., Shenoy, S. R., Chan, P. K. S., McMahon, J. B., et al. (2010). Broad-spectrum in vitro activity and in vivo efficacy of the antiviral protein griffithsin against emerging viruses of the family Coronaviridae. *J. Virol.* 84, 2511–2521. doi: 10.1128/JVI.02322-09

Olearo, F., Nguyen, H., Bonnet, F., Yerly, S., Wandeler, G., Stoeckle, M., et al. (2019). Impact of the M184V/I mutation on the efficacy of Abacavir/Lamivudine/Dolutegravir therapy in HIV treatment-experienced patients. *Open Forum Infect. Dis.* 6:ofz330. doi: 10.1093/ofid/ofz330

Parris, G. E. (2004). Hypothesis links emergence of chloroquine-resistant malaria and other intracellular pathogens and suggests a new strategy for treatment of diseases caused by intracellular parasites. *Med. Hypotheses* 62, 354–357. doi: 10.1016/j.mehy.2003.12.004

Payne, S. (2017). "Chapter 17 - Family Coronaviridae," in *Viruses*, ed. S. Payne (Cambridge, MA: Academic Press), 149–158. doi: 10.1016/B978-0-12-803109-4.00017-9

Pedersen, N. C., Black, J. W., Boyle, J. F., Evermann, J. F., McKeirnan, A. J., and Ott, R. L. (1984). "Pathogenic differences between various feline coronavirus isolates," in *Molecular Biology and Pathogenesis of Coronaviruses Advances in Experimental Medicine and Biology*, eds P. J. M. Rottier, B. A. M. van der Zeijst, W. J. M. Spaan, and M. C. Horzinek (Boston, MA: Springer), 365–380. doi: 10.1007/978-1-4615-9373-7_36

Peters, H. L., Jochmans, D., de Wilde, A. H., Posthuma, C. C., Snijder, E. J., Neyts, J., et al. (2015). Design, synthesis and evaluation of a series of acyclic fleximer nucleoside analogues with anti-coronavirus activity. *Bioorg. Med. Chem. Lett.* 25, 2923–2926. doi: 10.1016/j.bmcl.2015.05.039

Pettitt, J., Zeitlin, L., Kim, D. H., Working, C., Johnson, J. C., Bohorov, O., et al. (2013). Therapeutic intervention of ebola virus infection in rhesus macaques

with the MB-003 monoclonal antibody cocktail. *Sci. Transl. Med.* 5, ra113–ra199. doi: 10.1126/scitranslmed.3006608

Pfefferle, S., Schöpf, J., Kögl, M., Friedel, C. C., Müller, M. A., Carbajo-Lozoya, J., et al. (2011). The SARS-Coronavirus-host interactome: identification of cyclophilins as target for pan-coronavirus inhibitors. *PLoS Pathog.* 7:e1002331. doi: 10.1371/journal.ppat.1002331

Prabakaran, P., Xiao, X., and Dimitrov, D. S. (2004). A model of the ACE2 structure and function as a SARS-CoV receptor. *Biochem. Biophys. Res. Commun.* 314, 235–241. doi: 10.1016/j.bbrc.2003.12.081

Preobrazhenskaya, M. N., and Olsufyeva, E. N. (2004). Patents on glycopeptides of the vancomycin family and their derivatives as antimicrobials: January 1999 – June 2003. *Expert Opin. Ther. Pat.* 14, 141–173. doi: 10.1517/13543776.14.2.141

Printsevskaya, S. S., Solovieva, S. E., Olsufyeva, E. N., Mirchink, E. P., Isakova, E. B., De Clercq, E., et al. (2005). Structure-activity relationship studies of a series of antiviral and antibacterial aglycon derivatives of the glycopeptide antibiotics vancomycin, eremomycin, and dechloroeremomycin. *J. Med. Chem.* 48, 3885–3890. doi: 10.1021/jm0500774

Proskurnina, E. V., Izmailov, D. Y., Sozarukova, M. M., Zhuravleva, T. A., Leneva, I. A., and Poromov, A. A. (2020). Antioxidant potential of antiviral drug umifenovir. *Molecules* 25:1577. doi: 10.3390/molecules25071577

PubChem (2005a). *Cyclosporin A*. Available online at: https://pubchem.ncbi.nlm.nih.gov/compound/5284373 (accessed April 1, 2020).

PubChem (2005b). *Mycophenolic Acid*. Available online at: https://pubchem.ncbi.nlm.nih.gov/compound/446541 (accessed April 1, 2020).

PubChem. (2005c). *Ribavirin*. Available online at: https://pubchem.ncbi.nlm.nih.gov/compound/37542 (accessed April 1, 2020).

Pukrittayakamee, S., Tarning, J., Jittamala, P., Charunwatthana, P., Lawpoolsri, S., Lee, S. J., et al. (2014). Pharmacokinetic Interactions between Primaquine and Chloroquine. *Antimicrob. Agents Chemother.* 58, 3354–3359. doi: 10.1128/AAC.02794-13

Pyrc, K., Bosch, B. J., Berkhout, B., Jebbink, M. F., Dijkman, R., Rottier, P., et al. (2006). Inhibition of human coronavirus NL63 infection at early stages of the replication cycle. *Antimicrob. Agents Chemother.* 50, 2000–2008. doi: 10.1128/AAC.01598-05

Rabi, F. A., Al Zoubi, M. S., Kasasbeh, G. A., Salameh, D. M., and Al-Nasser, A. D. (2020). SARS-CoV-2 and Coronavirus disease 2019: what we know so far. *Pathog. Basel Switz.* 9:231. doi: 10.3390/pathogens9030231

Ramajayam, R., Tan, K.-P., Liu, H.-G., and Liang, P.-H. (2010). Synthesis, docking studies, and evaluation of pyrimidines as inhibitors of SARS-CoV 3CL protease. *Bioorg. Med. Chem. Lett.* 20, 3569–3572. doi: 10.1016/j.bmcl.2010.04.118

Ramos-Tovar, E., and Muriel, P. (2019). "Chapter 9 - phytotherapy for the liver," in *Dietary Interventions in Liver Disease*, eds R. R. Watson and V. R. Preedy (Cambridge, MA: Academic Press), 101–121. doi: 10.1016/B978-0-12-814466-4.00009-4

Ranieri, V. M., Pettilä, V., Karvonen, M. K., Jalkanen, J., Nightingale, P., Brealey, D., et al. (2020). Effect of intravenous interferon β-1a on death and days free from mechanical ventilation among patients with moderate to severe acute respiratory distress syndrome: a randomized clinical trial. *JAMA* 323, 725–733. doi: 10.1001/jama.2019.22525

Reguera, J., Santiago, C., Mudgal, G., Ordoño, D., Enjuanes, L., and Casasnovas, J. M. (2012). Structural bases of coronavirus attachment to host aminopeptidase n and its inhibition by neutralizing antibodies. *PLoS Pathog.* 8:e1002859. doi: 10.1371/journal.ppat.1002859

Reusken, C. B., Raj, V. S., Koopmans, M. P., and Haagmans, B. L. (2016). Cross host transmission in the emergence of MERS coronavirus. *Curr. Opin. Virol.* 16, 55–62. doi: 10.1016/j.coviro.2016.01.004

Roback, J. D., and Guarner, J. (2020). Convalescent plasma to Treat COVID-19: possibilities and challenges. *JAMA* 323, 1561–1562. doi: 10.1001/jama.2020.4940

Roberts, R. S., Yount, B. L., Sims, A. C., Baker, S., and Baric, R. S. (2006). "Renilla luciferase as a reporter to assess SARS-CoV mRNA transcription regulation and efficacy of ANTI-SARS-CoV agents," in *The Nidoviruses Advances in Experimental Medicine and Biology*, eds S. Perlman and K. V. Holmes (Boston, MA: Springer), 597–600. doi: 10.1007/978-0-387-33012-9_108

Roques, P., Thiberville, S.-D., Dupuis-Maguiraga, L., Lum, F.-M., Labadie, K., Martinon, F., et al. (2018). Paradoxical effect of chloroquine treatment in enhancing chikungunya virus infection. *Viruses* 10:268. doi: 10.3390/v10050268

Rossignol, J.-F. (2014). Nitazoxanide: a first-in-class broad-spectrum antiviral agent. *Antiviral Res.* 110, 94–103. doi: 10.1016/j.antiviral.2014.07.014

Rossignol, J.-F. (2016). Nitazoxanide, a new drug candidate for the treatment of Middle East respiratory syndrome coronavirus. *J. Infect. Public Health* 9, 227–230. doi: 10.1016/j.jiph.2016.04.001

Ruiz-Irastorza, G., Ramos-Casals, M., Brito-Zeron, P., and Khamashta, M. A. (2010). Clinical efficacy and side effects of antimalarials in systemic lupus erythematosus: a systematic review. *Ann. Rheum. Dis.* 69, 20–28. doi: 10.1136/ard.2008.101766

Saijo, M., Morikawa, S., Fukushi, S., Mizutani, T., Hasegawa, H., Nagata, N., et al. (2005). Inhibitory effect of mizoribine and ribavirin on the replication of severe acute respiratory syndrome (SARS)-associated coronavirus. *Antiviral Res.* 66, 159–163. doi: 10.1016/j.antiviral.2005.01.003

Sallard, E., Lescure, F.-X., Yazdanpanah, Y., Mentre, F., and Peiffer-Smadja, N. (2020). Type 1 interferons as a potential treatment against COVID-19. *Antiviral Res.* 178:104791. doi: 10.1016/j.antiviral.2020.104791

Samuel, C. E. (2001). Antiviral Actions of Interferons. *Clin. Microbiol. Rev.* 14, 778–809. doi: 10.1128/CMR.14.4.778-809.2001

Sanan-Mishra, N., Chakraborty, S., Gupta, D., and Mukherjee, S. K. (2017). "RNAi Suppressors: biology and Mechanisms," in *Plant Epigenetics RNA Technologies*, eds N. Rajewsky, S. Jurga, and J. Barciszewski (Cham: Springer International Publishing), 199–230. doi: 10.1007/978-3-319-55520-1_11

Sayce, A. C., Miller, J. L., and Zitzmann, N. (2010). Targeting a host process as an antiviral approach against dengue virus. *Trends Microbiol.* 18, 323–330. doi: 10.1016/j.tim.2010.04.003

Scheller, J., and Rose-John, S. (2006). Interleukin-6 and its receptor: from bench to bedside. *Med. Microbiol. Immunol.* 195, 173–183. doi: 10.1007/s00430-006-0019-9

Schwarz, S., Wang, K., Yu, W., Sun, B., and Schwarz, W. (2011). Emodin inhibits current through SARS-associated coronavirus 3a protein. *Antiviral Res.* 90, 64–69. doi: 10.1016/j.antiviral.2011.02.008

Shah, P. P., Wang, T., Kaletsky, R. L., Myers, M. C., Purvis, J. E., Jing, H., et al. (2010). A small-molecule oxocarbazate inhibitor of human cathepsin L blocks severe acute respiratory syndrome and ebola pseudotype virus infection into human embryonic kidney 293T cells. *Mol. Pharmacol.* 78, 319–324. doi: 10.1124/mol.110.064261

Shah, P. S., and Schaffer, D. V. (2011). Antiviral RNAi: translating science towards therapeutic success. *Pharm. Res.* 28, 2966–2982. doi: 10.1007/s11095-011-0549-8

Shanmugaraj, B., Siriwattananon, K., Wangkanont, K., and Phoolcharoen, W. (2020). Perspectives on monoclonal antibody therapy as potential therapeutic intervention for Coronavirus disease-19 (COVID-19). *Asian Pac. J. Allergy Immunol.* 38, 10–18. doi: 10.12932/AP-200220-0773

Sheahan, T. P., Sims, A. C., Graham, R. L., Menachery, V. D., Gralinski, L. E., Case, J. B., et al. (2017). Broad-spectrum antiviral GS-5734 inhibits both epidemic and zoonotic coronaviruses. *Sci. Transl. Med.* 9:eaal3653. doi: 10.1126/scitranslmed.aal3653

Sheahan, T. P., Sims, A. C., Leist, S. R., Schäfer, A., Won, J., Brown, A. J., et al. (2020a). Comparative therapeutic efficacy of remdesivir and combination lopinavir, ritonavir, and interferon beta against MERS-CoV. *Nat. Commun.* 11, 1–14. doi: 10.1038/s41467-019-13940-6

Sheahan, T. P., Sims, A. C., Zhou, S., Graham, R. L., Pruijssers, A. J., Agostini, M. L., et al. (2020b). An orally bioavailable broad-spectrum antiviral inhibits SARS-CoV-2 in human airway epithelial cell cultures and multiple coronaviruses in mice. *Sci. Transl. Med.* 6:eabb5883. doi: 10.1126/scitranslmed.abb5883

Shen, L., Yang, Y., Ye, F., Liu, G., Desforges, M., Talbot, P. J., et al. (2016). Safe and sensitive antiviral screening platform based on recombinant human Coronavirus OC43 expressing the luciferase reporter gene. *Antimicrob. Agents Chemother.* 60, 5492–5503. doi: 10.1128/AAC.00814-16

Shi, Y., Yang, D. H., Xiong, J., Jia, J., Huang, B., and Jin, Y. X. (2005). Inhibition of genes expression of SARS coronavirus by synthetic small interfering RNAs. *Cell Res.* 15, 193–200. doi: 10.1038/sj.cr.7290286

Shie, J.-J., Fang, J.-M., Kuo, T.-H., Kuo, C.-J., Liang, P.-H., Huang, H.-J., et al. (2005). Inhibition of the severe acute respiratory syndrome 3CL protease by peptidomimetic α,β-unsaturated esters. *Bioorg. Med. Chem.* 13, 5240–5252. doi: 10.1016/j.bmc.2005.05.065

Shin, J. S., Jung, E., Kim, M., Baric, R. S., and Go, Y. Y. (2018). Saracatinib inhibits middle east respiratory syndrome-coronavirus replication in vitro. *Viruses* 10:283. doi: 10.3390/v10060283

Siu, Y. L., Teoh, K. T., Lo, J., Chan, C. M., Kien, F., Escriou, N., et al. (2008). The M, E, and N structural proteins of the severe acute respiratory syndrome coronavirus are required for efficient assembly, trafficking, and release of virus-like particles. *J. Virol.* 82, 11318–11330. doi: 10.1128/JVI.01052-08

Smith, J. (2020). *CureVac Bids to Develop First mRNA Coronavirus Vaccine*. Available online at: https://www.labiotech.eu/medical/curevac-coronavirus-outbreak-cepi/ (accessed June 8, 2020)

Sperber, S. J., and Hayden, F. G. (1989). Comparative susceptibility of respiratory viruses to recombinant interferons-α2b and -β. *J. Interferon Res.* 9, 285–293. doi: 10.1089/jir.1989.9.285

Stocks, P. A., Raynes, K. J., Bray, P. G., Park, B. K., O'Neill, P. M., and Ward, S. A. (2002). Novel short chain chloroquine analogues retain activity against chloroquine resistant K1 plasmodium falciparum. *J. Med. Chem.* 45, 4975–4983. doi: 10.1021/jm0108707

Struck, A.-W., Axmann, M., Pfefferle, S., Drosten, C., and Meyer, B. (2012). A hexapeptide of the receptor-binding domain of SARS corona virus spike protein blocks viral entry into host cells via the human receptor ACE2. *Antiviral Res.* 94, 288–296. doi: 10.1016/j.antiviral.2011.12.012

Sun, C., Chen, L., Yang, J., Luo, C., Zhang, Y., Li, J., et al. (2020). SARS-CoV-2 and SARS-CoV Spike-RBD Structure and Receptor Binding Comparison and Potential Implications on Neutralizing Antibody and Vaccine Development. *bioRxiv [Preprint]* doi: 10.1101/2020.02.16.951723

Tai, W., He, L., Zhang, X., Pu, J., Voronin, D., Jiang, S., et al. (2020). Characterization of the receptor-binding domain (RBD) of 2019 novel coronavirus: implication for development of RBD protein as a viral attachment inhibitor and vaccine. *Cell. Mol. Immunol.* 17, 613–620. doi: 10.1038/s41423-020-0400-4

Takano, T., Katoh, Y., Doki, T., and Hohdatsu, T. (2013). Effect of chloroquine on feline infectious peritonitis virus infection in vitro and in vivo. *Antiviral Res.* 99, 100–107. doi: 10.1016/j.antiviral.2013.04.016

Takashita, E. (2020). Influenza polymerase inhibitors: mechanisms of action and resistance. *Cold Spring Harb. Perspect. Med.* 2:a038687. doi: 10.1101/cshperspect.a038687

Tan, E. L. C., Ooi, E. E., Lin, C.-Y., Tan, H. C., Ling, A. E., Lim, B., et al. (2004). Inhibition of SARS Coronavirus infection in vitro with clinically approved antiviral drugs. *Emerg. Infect. Dis.* 10, 581–586. doi: 10.3201/eid1004.030458

Tanner, J. A., Zheng, B.-J., Zhou, J., Watt, R. M., Jiang, J.-Q., Wong, K.-L., et al. (2005). The adamantane-derived bananins are potent inhibitors of the helicase activities and replication of SARS Coronavirus. *Chem. Biol.* 12, 303–311. doi: 10.1016/j.chembiol.2005.01.006

Tay, M. Y. F., Fraser, J. E., Chan, W. K. K., Moreland, N. J., Rathore, A. P., Wang, C., et al. (2013). Nuclear localization of dengue virus (DENV) 1–4 non-structural protein 5; protection against all 4 DENV serotypes by the inhibitor Ivermectin. *Antiviral Res.* 99, 301–306. doi: 10.1016/j.antiviral.2013.06.002

Thomas, S. M., and Brugge, J. S. (1997). Cellular functions regulated by Src family kinases. *Annu. Rev. Cell Dev. Biol.* 13, 513–609. doi: 10.1146/annurev.cellbio.13.1.513

Thomas, T. (2020). *The Oxford Vaccine Centre COVID-19 Phase II/III Clinical Trial Explained. COVID-19 Oxf. Vaccine Trial*. Available online at: https://covid19vaccinetrial.co.uk/phase-iiiii-trial-explained (accessed July 1, 2020).

Toniati, P., Piva, S., Cattalini, M., Garrafa, E., Regola, F., Castelli, F., et al. (2020). Tocilizumab for the treatment of severe COVID-19 pneumonia with hyperinflammatory syndrome and acute respiratory failure: a single center study of 100 patients in Brescia. Italy. *Autoimmun. Rev.* 19:102568. doi: 10.1016/j.autrev.2020.102568

Totura, A. L., and Bavari, S. (2019). Broad-spectrum coronavirus antiviral drug discovery. *Expert Opin. Drug Discov.* 14, 397–412. doi: 10.1080/17460441.2019.1581171

Tu, Y.-F., Chien, C.-S., Yarmishyn, A. A., Lin, Y.-Y., Luo, Y.-H., Lin, Y.-T., et al. (2020). A review of SARS-CoV-2 and the ongoing clinical trials. *Int. J. Mol. Sci.* 21:2657. doi: 10.3390/ijms21072657

Ullah, H., Hou, W., Dakshanamurthy, S., and Tang, Q. (2019). Host targeted antiviral (HTA): functional inhibitor compounds of scaffold protein RACK1 inhibit herpes simplex virus proliferation. *Oncotarget* 10, 3209–3226. doi: 10.18632/oncotarget.26907

Unübol, M., Ayhan, M., and Guney, E. (2011). Hypoglycemia induced by hydroxychloroquine in a patient treated for rheumatoid arthritis. *J. Clin. Rheumatol. Pract. Rep. Rheum. Musculoskelet. Dis.* 17, 46–47. doi: 10.1097/RHU.0b013e3182098e1f

Ursing, J., Rombo, L., Eksborg, S., Larson, L., Bruvoll, A., Tarning, J., et al. (2020). High-dose chloroquine for uncomplicated plasmodium falciparum malaria is well tolerated and causes similar QT interval prolongation as standard-dose chloroquine in children. *Antimicrob. Agents Chemother.* 64:e01846-19.doi: 10.1128/AAC.01846-19

van Doremalen, N., Bushmaker, T., Morris, D. H., Holbrook, M. G., Gamble, A., Williamson, B. N., et al. (2020). Aerosol and surface stability of SARS-CoV-2 as compared with SARS-CoV-1. *N. Engl. J. Med.* 382, 1564–1567. doi: 10.1056/NEJMc2004973

Varghese, F. S., Kaukinen, P., Gläsker, S., Bespalov, M., Hanski, L., Wennerberg, K., et al. (2016). Discovery of berberine, abamectin and ivermectin as antivirals against chikungunya and other alphaviruses. *Antiviral Res.* 126, 117–124. doi: 10.1016/j.antiviral.2015.12.012

Vassão, R. C., de Franco, M. T., Hartz, D., Modolell, M., Sippel, A. E., and Pereira, C. A. (2000). Down-regulation of bgp1a viral receptor by interferon-γ Is related to the antiviral state and resistance to mouse hepatitis virus 3 infection. *Virology* 274, 278–283. doi: 10.1006/viro.2000.0463

Villegas-Rosales, P. M., Méndez-Tenorio, A., Ortega-Soto, E., and Barrón, B. L. (2012). Bioinformatics prediction of siRNAs as potential antiviral agents against dengue viruses. *Bioinformation* 8, 519–522. doi: 10.6026/97320630008519

Vincent, M. J., Bergeron, E., Benjannet, S., Erickson, B. R., Rollin, P. E., Ksiazek, T. G., et al. (2005). Chloroquine is a potent inhibitor of SARS coronavirus infection and spread. *Virol. J.* 2:69. doi: 10.1186/1743-422X-2-69

Vuong, W., Khan, M. B., Fischer, C., Arutyunova, E., Lamer, T., Shields, J., et al. (2020). Feline coronavirus drug inhibits the main protease of SARS-CoV-2 and blocks virus replication. *bioRxiv [Preprint]* doi: 10.1101/2020.05.03.073080

Walls, A. C., Park, Y.-J., Tortorici, M. A., Wall, A., McGuire, A. T., and Veesler, D. (2020). Structure, function, and antigenicity of the SARS-CoV-2 spike glycoprotein. *Cell* 181, 281e6–292.e6. doi: 10.1016/j.cell.2020.02.058

Wang, C., Li, W., Drabek, D., Okba, N. M. A., van Haperen, R., Osterhaus, A. D. M. E., et al. (2020a). A human monoclonal antibody blocking SARS-CoV-2 infection. *Nat. Commun.* 11:2251. doi: 10.1038/s41467-020-16256-y

Wang, M., Cao, R., Zhang, L., Yang, X., Liu, J., Xu, M., et al. (2020b). Remdesivir and chloroquine effectively inhibit the recently emerged novel coronavirus (2019-nCoV) in vitro. *Cell Res.* 30:269–271. doi: 10.1038/s41422-020-0282-0

Wang, Y., Jiang, W., He, Q., Wang, C., Wang, B., Zhou, P., et al. (2020c). Early, low-dose and short-term application of corticosteroid treatment in patients with severe COVID-19 pneumonia: single-center experience from Wuhan, China. *medRxiv [Preprint]* doi: 10.1101/2020.03.06.20032342

Wang, Y., Zhang, D., Du, G., Du, R., Zhao, J., Jin, Y., et al. (2020d). Remdesivir in adults with severe COVID-19: a randomised, double-blind, placebo-controlled, multicentre trial. *Lancet* 395, 1569–1578. doi: 10.1016/S0140-6736(20)31022-9

Wang, Y., Sun, Y., Wu, A., Xu, S., Pan, R., Zeng, C., et al. (2015). Coronavirus nsp10/nsp16 methyltransferase can be targeted by nsp10-derived peptide in vitro and in vivo to reduce replication and pathogenesis. *J. Virol.* 89, 8416–8427. doi: 10.1128/JVI.00948-15

Warren, T. K., Jordan, R., Lo, M. K., Ray, A. S., Mackman, R. L., Soloveva, V., et al. (2016). Therapeutic efficacy of the small molecule GS-5734 against Ebola virus in rhesus monkeys. *Nature* 531, 381–385. doi: 10.1038/nature17180

Weiss, S. R., and Navas-Martin, S. (2005). Coronavirus pathogenesis and the emerging pathogen severe acute respiratory syndrome Coronavirus. *Microbiol. Mol. Biol. Rev.* 69, 635–664. doi: 10.1128/MMBR.69.4.635-664.2005

Wen, C. C., Kuo, Y. H., Jan, J. T., Liang, P. H., Wang, S. Y., Liu, H. G., et al. (2007). Specific plant terpenoids and lignoids possess potent antiviral activities against severe acute respiratory syndrome coronavirus. *J. Med. Chem.* 50, 4087–4095. doi: 10.1021/jm070295s

WHO (2020a). *Clinical management of COVID-19*. Available online at: https://www.who.int/publications-detail-redirect/clinical-management-of-covid-19 (accessed June 8, 2020)

WHO (2020b). *Novel Coronavirus (2019-nCoV) Situation Reports*. Available online at: https://www.who.int/emergencies/diseases/novel-coronavirus-2019/situation-reports (accessed July 04, 2020)

WHO (2020c). *Q&A: Hydroxychloroquine and COVID-19*. Available online at: https://www.who.int/news-room/q-a-detail/q-a-hydroxychloroquine-and-covid-19 (accessed July 5, 2020)

Wilkinson, J. R., Lane, S. J., and Lee, T. H. (1991). Effects of corticosteroids on cytokine generation and expression of activation antigens by monocytes in bronchial asthma. *Int. Arch. Allergy Immunol.* 94, 220–221. doi: 10.1159/000235365

Wilson, R. C., and Doudna, J. A. (2013). Molecular mechanisms of RNA interference. *Annu. Rev. Biophys.* 42, 217–239. doi: 10.1146/annurev-biophys-083012-130404

Wrapp, D., Wang, N., Corbett, K. S., Goldsmith, J. A., Hsieh, C.-L., Abiona, O., et al. (2020). Cryo-EM structure of the 2019-nCoV spike in the prefusion conformation. *Science* 367:1260. doi: 10.1126/science.abb2507

Wu, C.-J., Jan, J.-T., Chen, C.-M., Hsieh, H.-P., Hwang, D.-R., Liu, H.-W., et al. (2004). Inhibition of severe acute respiratory syndrome coronavirus replication by niclosamide. *Antimicrob. Agents Chemother.* 48, 2693–2696. doi: 10.1128/AAC.48.7.2693-2696.2004

Wu, D., Wu, T., Liu, Q., and Yang, Z. (2020). The SARS-CoV-2 outbreak: what we know. *Int. J. Infect. Dis.* 94, 44–48. doi: 10.1016/j.ijid.2020.03.004

Xu, X., Han, M., Li, T., Sun, W., Wang, D., Fu, B., et al. (2020). Effective treatment of severe COVID-19 patients with tocilizumab. *Proc. Natl. Acad. Sci. U.S.A.* 117:202005615. doi: 10.1073/pnas.2005615117

Yan, R., Zhang, Y., Li, Y., Xia, L., Guo, Y., and Zhou, Q. (2020). Structural basis for the recognition of the SARS-CoV-2 by full-length human ACE2. *Science* 367, 1444–1448. doi: 10.1126/science.abb2762

Yang, D., and Leibowitz, J. L. (2015). The structure and functions of coronavirus genomic 3' and 5' ends. *Virus Res.* 206, 120–133. doi: 10.1016/j.virusres.2015.02.025

Yao, T.-T., Qian, J.-D., Zhu, W.-Y., Wang, Y., and Wang, G.-Q. (2020a). A systematic review of lopinavir therapy for SARS coronavirus and MERS coronavirus—A possible reference for coronavirus disease-19 treatment option. *J. Med. Virol.* 92, 556–563. doi: 10.1002/jmv.25729 n/a.,

Yao, X., Ye, F., Zhang, M., Cui, C., Huang, B., Niu, P., et al. (2020b). In vitro antiviral activity and projection of optimized dosing design of hydroxychloroquine for the treatment of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). *Clin. Infect. Dis.* 9:ciaa237. doi: 10.1093/cid/ciaa237

Yogo, Y., Hirano, N., Hino, S., Shibuta, H., and Matumoto, M. (1977). Polyadenylate in the Virion RNA of Mouse Hepatitis Virus. *J. Biochem.* 82, 1103–1108. doi: 10.1093/oxfordjournals.jbchem.a131782

Zha, L., Li, S., Pan, L., Tefsen, B., Li, Y., French, N., et al. (2020). Corticosteroid treatment of patients with coronavirus disease 2019 (COVID-19). *Med. J. Aust.* 212, 416–420. doi: 10.5694/mja2.50577

Zhang, C., Wu, Z., Li, J., Zhao, H., and Wang, G. (2020a). Cytokine release syndrome in severe COVID-19: interleukin-6 receptor antagonist tocilizumab may be the key to reduce mortality. *Int. J. Antimicrob. Agents* 55:105954. doi: 10.1016/j.ijantimicag.2020.105954

Zhang, S., Li, L., Shen, A., Chen, Y., and Qi, Z. (2020b). Rational use of tocilizumab in the treatment of novel coronavirus pneumonia. *Clin. Drug Investig.* 26, 1–8. doi: 10.1007/s40261-020-00917-3

Zhang, H.-Z., Zhang, H., Kemnitzer, W., Tseng, B., Cinatl, J., Michaelis, M., et al. (2006). Design and synthesis of dipeptidyl glutaminyl fluoromethyl ketones as potent severe acute respiratory syndrome coronovirus (SARS-CoV) inhibitors. *J. Med. Chem.* 49, 1198–1201. doi: 10.1021/jm0507678

Zhu, Z., Chakraborti, S., He, Y., Roberts, A., Sheahan, T., Xiao, X., et al. (2007). Potent cross-reactive neutralization of SARS coronavirus isolates by human monoclonal antibodies. *Proc. Natl. Acad. Sci. U.S.A.* 104, 12123–12128. doi: 10.1073/pnas.0701000104

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

OPINION
published: 14 August 2020
doi: 10.3389/fimmu.2020.02051

Vaccine Development Against COVID-19 Prior to Pandemic Outbreaks, Using *in vitro* Evolution and Reverse Genetics

*Hatem Zayed**

Department of Biomedical Sciences, College of Health Sciences, QU Health, Qatar University, Doha, Qatar

Keywords: VLP vaccines, COVID-19, pandemic outbreak, SARS-CoV2, integrating vectors, coronaviruses

The coronavirus disease 2019 (COVID-19) is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which is an enveloped, non-segmented, positive-sense RNA virus (1). The complete genome of SARS-CoV-2 is 29.9 kb (2, 3). The virus genome contains four essential proteins that are believed to be important for the infectious ability of the virus, the glycoprotein spike (S), nucleocapsid (N), matrix (M), and small envelope (E) proteins (4). The S glycoprotein, which mediates entry of the virus into the target cells, is the main target for host defense antibodies (5).

As of May, 2020, the COVID-19 pandemic has spread to 213 countries and territories worldwide with nearly 6 million confirmed cases and ∼6% mortality (who.int). As the outbreaks spread, scientists across the globe are racing to develop vaccines against COVID-19. Since coronaviruses are increasing alarmingly, there is an urgent need for a safe and effective vaccine to prevent the spread of the virus during pandemic outbreaks, and stop deaths associated with the virulent COVID-19. However, developing vaccines that are safe and effective requires a lot of time and testing. It is estimated that 18 months are needed to develop such a vaccine.

Although it is challenging to predict the severity, time, and location of future coronavirus pandemics, we can be prepared for the highly pathogenic strains that are likely to reemerge and cause future pandemics. This can be done using previous epidemiological studies on coronaviruses. For example, in 2019, Chinese scientists anticipated that there would be a potential bat coronavirus that would likely emerge and infect humans, and might cause an imminent outbreak in China (6). Unfortunately, the efforts of these Chinese scientists were met with no interest from the Chinese government, evidenced by the lack of proper preparation for the current pandemic when it appeared in China a few months ago. We now know that SARS-CoV-2 shares 88% identity with two SARS-like coronaviruses (bat-SL-CoVZXC21 and bat-SL-CoVZC45) that both originated in China, and use the same human angiotensin-converting enzyme 2 receptor for cell entry during the process of infection (3). If we had reacted to these predictions, then we would very likely have avoided the current crisis. In response to such forewarnings from scientists, a predictive vaccine could have been designed and developed for the potential virus pandemic. Developing a vaccine during or after the pandemic outbreaks is too slow to provide timely responses against COVID-19, and risks many lives. Producing an efficient and safe vaccine ready for human use can take up to 18 months, according to the World Health Organization (WHO). Therefore, anticipating the virus mutations responsible for the possible reemergence of highly pathogenic virulent strains may be a means by which to prepare for future, newly emerging, pandemic strains.

The process of preparing a predictive vaccine can be summarized as follows: (1) The SARS-CoV-2 genome would be used as a template for *in vitro* evolution through DNA

OPEN ACCESS

Edited by:
Ata Murat Kaynar, University of Pittsburgh School of Medicine, United States

Reviewed by:
Selidji Todagbe AGNANDJI, Centre de Recherche Médicales de Lambaréné, Gabon

***Correspondence:**
Hatem Zayed
hatem.zayed@qu.edu.qa

Specialty section:
This article was submitted to Vaccines and Molecular Therapeutics, a section of the journal Frontiers in Immunology

Received: *14 April 2020*
Accepted: *28 July 2020*
Published: *14 August 2020*

Citation:
Zayed H (2020) Vaccine Development Against COVID-19 Prior to Pandemic Outbreaks, Using in vitro Evolution and Reverse Genetics. Front. Immunol. 11:2051. doi: 10.3389/fimmu.2020.02051

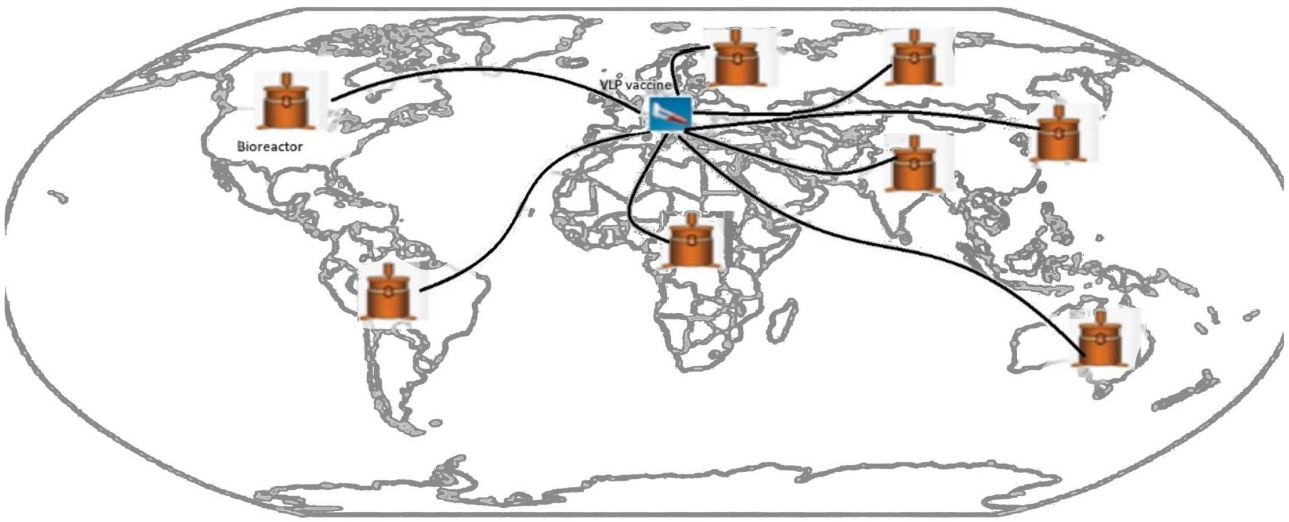


FIGURE 1 | The possible satellite WHO-supervised distributions sites of the preemptive VLP vaccine during COVID-19 pandemic.

shuffling techniques (7, 8). Random recombination of a viral genome in a test tube mimics the possible assortment and mutations that occur in the virus in nature, creating all possible random recombination. These changes could be incorporated into the four essential viral genes (S, N, M, and E). (2) These genes would then be subcloned individually into integrating gene delivery vehicles, such as lentiviral vectors (9) or transposons (10, 11). (3) Using reverse genetic strategies (12, 13), the recombinant constructs would be transfected into cell lines susceptible to coronaviruses (14), leading to secretion of virus-like particles (VLPs) from the cells into the culture media. (4) The recombinant VLPs could then be harvested and purified from the supernatant of the culture media. (5) VLPs would then be tested for proper assembly and integrity using electron microscopy and different methods of protein quantification. (6) All possible mutant VLPs would be tested using different functional assays to check for possible antigenicity. (7) The candidate VLPs proven to be functional and highly immunogenic could then be used in challenge experiments using animal models and recombinant live virulent viruses believed to be highly pathogenic. (8) The VLPs that are highly protective against the highly pathogenic recombinant strains would be further selected and stable cell lines made from all candidate VLP vaccines. (9) These cell lines could be expanded using bioreactors and stored for further use. Lentiviral vectors could generate a stable cell line that is transgenic for the highly immunogenic antigenic determinants of COVID-19 (15), and would be able to continuously secrete VLPs into the culture media (16). Thereafter, during the time of pandemic, suitable stored transgenic cell lines could be used, based on the reemergent pandemic viral mutant strain, and could be easily shipped across the globe, thawed, and manufactured on a large scale in customized large-sized bioreactors (**Figure 1**). VLP vaccines could be used as therapeutic vaccines and administered to infected individuals (17), or as vaccines into healthy non-infected individuals. The immunodominant epitopes (18) of the viral mutants specific for the virus would elicit potent immune responses that could be life-saving (19). The genomeless hollow shells would mimic the actual live virus in terms of eliciting a strong immune response; however, these shells are neither replicative nor infectious by themselves (20).

Such a project should be done through international collaborations and under the supervision of the WHO. Stocks of these VLP vaccines could be stored as vials of transgenic cell lines, able to be regularly expanded and checked for their quality and ability to generate VLP vaccines. Stocks of these vials could be kept in different countries with satellite distributors managed and administered by the WHO. This project would require scientists with high degrees of skill that are trained in the field of vaccine design and development, and trained in several other fields such as molecular biology, virology, infectious diseases, and cell biology.

The development of VLP vaccines against reemerging viral pandemics would be far affordable than the economic costs of the current COVID-19 pandemic. Such project requires concerted global efforts of multiple organizations, which is expected to save thousands of lives. I do believe that the time has come for all government officials and policymakers to listen very carefully to science and scientists' recommendations to ensure the health and well-being of people of our planet.

Abbreviations: COVID-19, coronavirus disease 2019; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; VLP, virus-like particle; WHO, World Health Organization.

AUTHOR CONTRIBUTIONS

HZ conceptualized the study and wrote the manuscript.

REFERENCES

1. Zhu N, Zhang D, Wang W, Li X, Yang B, Song J, et al. A novel coronavirus from patients with pneumonia in China, 2019. *N Engl J Med.* (2020) 382:727–33. doi: 10.1056/NEJMoa2001017
2. Wu F, Zhao S, Yu B, Chen YM, Wang W, Song ZG, et al. A new coronavirus associated with human respiratory disease in China. *Nature.* (2020) 579:265–9. doi: 10.1038/s41586-020-2008-3
3. Lu R, Zhao X, Li J, Niu P, Yang B, Wu H, et al. Genomic characterisation and epidemiology of 2019 novel coronavirus: implications for virus origins and receptor binding. *Lancet.* (2020) 395:565–74. doi: 10.1016/S0140-6736(20)30251-8
4. Cui J, Li F, Shi ZL. Origin and evolution of pathogenic coronaviruses. *Nat Rev Microbiol.* (2019) 17:181–92. doi: 10.1038/s41579-018-0118-9
5. Walls AC, Park YJ, Tortorici MA, Wall A, McGuire AT, Veesler D. Structure, function, and antigenicity of the SARS-CoV-2 spike glycoprotein. *Cell.* (2020) 181:281–92.e6. doi: 10.1101/2020.02.19.956581
6. Fan Y, Zhao K, Shi ZL, Zhou P. Bat coronaviruses in China. *Viruses.* (2019) 11:210. doi: 10.3390/v11030210
7. Kikuchi M, Harayama S. DNA shuffling and family shuffling for in vitro gene evolution. *Methods Mol Biol.* (2002) 182:243–57. doi: 10.1385/1-59259-194-9:243
8. Rigouin C, Nguyen HA, Schalk AM, Lavie A. Discovery of human-like L-asparaginases with potential clinical use by directed evolution. *Sci Rep.* (2017) 7:10224. doi: 10.1038/s41598-017-10758-4
9. McBurney SP, Young KR, Ross TM. Membrane embedded HIV-1 envelope on the surface of a virus-like particle elicits broader immune responses than soluble envelopes. *Virology.* (2007) 358:334–46. doi: 10.1016/j.virol.2006.08.032
10. de Wispelaere M, Ricklin M, Souque P, Frenkiel MP, Paulous S, Garcìa-Nicolàs O, et al. A lentiviral vector expressing japanese encephalitis virus-like particles elicits broad neutralizing antibody response in pigs. *PLoS Negl Trop Dis.* (2015) 9:e0004081. doi: 10.1371/journal.pntd.0004081
11. Ivics Z, Kaufman CD, Zayed H, Miskey C, Walisko O, Izsvák Z. The Sleeping Beauty transposable element: evolution, regulation and genetic applications. *Curr Issues Mol Biol.* (2004) 6:43–55. doi: 10.1016/j.ymthe.2003.11.009
12. Stobart CC, Moore ML. RNA virus reverse genetics and vaccine design. *Viruses.* (2014) 6:2531–50. doi: 10.3390/v6072531
13. Xu L, Bao L, Lau SY, Wu WL, Yuan J, Gu S, et al. Hemagglutinin amino acids related to receptor specificity could affect the protection efficacy of H5N1 and H7N9 avian influenza virus vaccines in mice. *Vaccine.* (2016) 34:2627–33. doi: 10.1016/j.vaccine.2016.03.031
14. Kaye M. SARS-associated coronavirus replication in cell lines. *Emerg Infect Dis.* (2006) 12:128–33. doi: 10.3201/eid1201.050496
15. Baranyi L, Doering CB, Denning G, Gautney RE, Harris KT, Spencer HT, et al. Rapid generation of stable cell lines expressing high levels of erythropoietin, factor VIII, and an antihuman CD20 antibody using lentiviral vectors. *Hum Gene Ther Methods.* (2013) 24:214–27. doi: 10.1089/hgtb.2013.002
16. Thompson CM, Aucoin MG, Kamen AA. Production of virus-like particles for vaccination. *Methods Mol Biol.* (2016) 1350:299–315. doi: 10.1007/978-1-4939-3043-2_14
17. Moingeon P, Almond J, de Wilde M. Therapeutic vaccines against infectious diseases. *Curr Opin Microbiol.* (2003) 6:462–71. doi: 10.1016/j.mib.2003.08.002
18. Ahmed SF, Quadeer AA, McKay MR. Preliminary identification of potential vaccine targets for the COVID-19 coronavirus (SARS-CoV-2) based on SARS-CoV immunological studies. *Viruses.* (2020) 12:254. doi: 10.1101/2020.02.03.933226
19. Fontana D, Etcheverrigaray M, Kratje R, Prieto C. Development of rabies virus-like particles for vaccine applications: production, characterization, and protection studies. *Methods Mol Biol.* (2016) 1403:155–66. doi: 10.1007/978-1-4939-3387-7_7
20. Muratori C, Bona R, Federico M. Lentivirus-based virus-like particles as a new protein delivery tool. *Methods Mol Biol.* (2010) 614:111–24. doi: 10.1007/978-1-60761-533-0_7

Conflict of Interest: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Public Health

ORIGINAL RESEARCH
published: 14 August 2020
doi: 10.3389/fpubh.2020.00477

Bibliometric Analysis on COVID-19: A Comparison of Research Between English and Chinese Studies

*Jingchun Fan[1,2†], Ya Gao[3,4†], Na Zhao[1], Runjing Dai[1], Hailiang Zhang[1], Xiaoyan Feng[1], Guoxiu Shi[1,5], Jinhui Tian[3,4], Che Chen[6], Brett D. Hambly[1,7] and Shisan Bao[1,7]**

[1] School of Public Health, Gansu University of Chinese Medicine, Lanzhou, China, [2] Center for Evidence-Based Medicine, Gansu University of Chinese Medicine, Lanzhou, China, [3] Evidence-Based Medicine Center, School of Basic Medical Sciences, Lanzhou University, Lanzhou, China, [4] Key Laboratory of Evidence-Based Medicine and Knowledge Translation of Gansu Province, Lanzhou, China, [5] School of Public Health, Lanzhou University, Lanzhou, China, [6] School of Clinical Medicine, Gansu University of Chinese Medicine, Lanzhou, China, [7] Discipline of Pathology, Charles Perkins Centre, Faculty of Medicine and Sciences, School of Medical Sciences, The University of Sydney, Sydney, NSW, Australia

OPEN ACCESS

Edited by:
Zisis Kozlakidis,
International Agency for Research on Cancer (IARC), France

Reviewed by:
Jernej Završnik,
Maribor Community Health Center, Slovenia
Peter Kokol,
University of Maribor, Slovenia
Carlos Mulet Forteza,
University of the Balearic Islands, Spain
Chichen Zhang,
Southern Medical University, China

***Correspondence:**
Shisan Bao
bob.bao@sydney.edu.au

[†] These authors have contributed equally to this work

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Public Health

Received: 31 May 2020
Accepted: 27 July 2020
Published: 14 August 2020

Citation:
Fan J, Gao Y, Zhao N, Dai R, Zhang H, Feng X, Shi G, Tian J, Chen C, Hambly BD and Bao S (2020) Bibliometric Analysis on COVID-19: A Comparison of Research Between English and Chinese Studies. Front. Public Health 8:477. doi: 10.3389/fpubh.2020.00477

Background: As an emerging infectious disease, COVID-19 has garnered great research interest. We aimed to explore the differences between English language and Chinese language Medical/Scientific journals publications, particularly aiming to explore the efficacy/contents of the literature published in English and Chinese in relation to the outcomes of management and characterization of COVID-19 during the early stage of COVID-19 pandemic.

Methods: Publications on COVID-19 research were retrieved from both English and Chinese databases. Bibliometric analyses were performed using VOSviewer 1.6.14, and CiteSpace V software. Network maps were generated to evaluate the collaborations between different authors, countries/provinces, and institutions.

Results: A total of 143 English and 721 Chinese original research articles and reviews on COVID-19 were included in our study. Most of the authors and institutions of the papers were from China before March 1st, 2020, however, the distribution of authors and institutions were mainly in developed countries or more wealthy areas of China. The range of the keywords in English publications was more extensive than those in Chinese. Traditional Chinese Medicine was seen more frequently in Chinese papers than in English. Of the 143 articles published in English, 54 articles were published by Chinese authors only and 21 articles were published jointly by Chinese and other overseas authors.

Conclusions: The publications in English have enabled medical practitioners and scientists to share/exchange information, while on the other hand, the publications in the Chinese language have provided complementary educational approaches for the local medical practitioners to understand the essential and key information to manage COVID-19 in the relatively remote regions of China, for the general population with a general level of education.

Keywords: bibliometric, COVID-19 outbreaks, SARS-CoV-2, English, Chinese

INTRODUCTION

The seriousness of the rapid spread of the SARS-CoV-2 virus has caused people to panic around the world since December 2019 (1). The tremendous danger of SARS-CoV-2, with a basic reproduction number (R_0) ranging from 2.30 to 3.58 (2), resulting in a pandemic with the number of infections reaching 9,653,048 to date (3). Consequently, considerable attention has been focused on COVID-19 from medical practitioners/scientists around the world to inhibit/stop the continuous transmission of SARS-CoV-2 and to develop guidelines for the effective treatment of severe cases.

To fight SARS-CoV-2, authorities in many countries have enforced social isolation restrictions to control the epidemic of COVID-19 throughout their countries, strategies utilized in China include wearing of facemasks in public areas, and minimizing outdoor, particularly mandating no public and/or private social gatherings (4, 5). The internet classes has allowed schools to continue to educate without classroom (6). Consequently, newly identified local COVID-19 cases have been reduced to near 0 in all of the provinces in China (7), mainly due to the active approaches outlined above, and strict limits to interstate/international travel. However, outside China, many countries now face escalating epidemics and are feeling overwhelmed due to the highly contagious nature of COVID-19.

As an emerging infectious disease, COVID-19 has garnered great research interest. Medical practitioners/scientists are studying the disease from various scientific and clinical areas, including specialists in infectious diseases, virology, microbiology. Many uncertainties remain as to certain epidemiological, seroepidemiological, clinical and virological characteristics of the virus and associated clinical features. The key task is to explore how to enhance host defenses and/or destroy viral resistance (8). Many researchers have published their data within top international, peer-reviewed, highly reputable journals, including NEJM, Lancet, Nature and Science (9). There are many studies that have been published in reputable Chinese journals (10, 11).

Bibliometrics used in the current study is to analysis quantitatively of citation scientific publications, based on constructing the citation graph, a network representing the citations of different documents. In addition, bibliometrics is also used for exploring comprehensively the impact of their field, a set of researchers, a particular paper within a specific field of research. Furthermore, VOSviewer software was used for constructing and visualizing bibliometric networks, whereas, CiteSpace V software was utilized for visualizing co-citation networks.

There are a few published papers, using bibliometric analysis of COVID-19, to explore the activity (12) and trends (13, 14) of COVID-19 research. We aimed to explore the differences between English language and Chinese language Medical/Scientific journals publications, particularly aiming to explore the efficacy/contents of the literature published in English and Chinese in relation to the outcomes of management and characterization of COVID-19 during the early stage of COVID-19 pandemic. We have undertaken a bibliometric comparison of research on COVID-19 between English and Chinese language journals.

MATERIALS AND METHODS

Data Source and Search Strategy

A comprehensive search was performed online using the English language databases Embase (15) and Scopus (16) on March 1, 2020, and simultaneously the Chinese databases Chinese Biomedical Database (SinoMed), CNKI, VIP and Wanfang were searched. The search terms were COVID-19, COVID 19, 2019-nCov, SARS-CoV-2, 2019 novel coronavirus, coronavirus disease 2019 and coronavirus disease-19. A detailed search strategy is presented in **Supplementary Figure 1**. The time period of publication was from 2019 to 1st March 2020. The search was performed on a single day to avoid bias caused by daily database updates. In the present study, only original articles and reviews published in either Chinese or English were included. The search retrieved 721 or 143 items in Chinese or English, respectively, that met the inclusion criteria.

Eligible Criteria

In the present study, only original articles and reviews, published in the Chinese or English languages were included. Studies including the following were excluded: (1) articles or reviews published on preprint sites such as bioRiov and medRiox; (2) translated versions of articles or reviews; (3) comments, editorials, and letters; (4) eliminating duplicate literature.

Study Selection and Data Management

Two reviewers independently performed study selection and data extraction. Differences of opinion were settled by consensus or referral to a third review author. Since some authors have the same short name, we added the affiliation behind the author names, if the same name's affiliation was different, it was considered as two different authors. For authors with more than one affiliation, we used the first one. For keywords with different expressions, we have processed them, leaving only one standardized keyword. We also reclassified publications from Hong Kong, Macau and Taiwan to China, and publications from England, Scotland, Northern Ireland, and Wales to the UK.

Data Analysis

Publication characteristics were tabulated, including titles, authors, co-cited authors, journal sources, keywords, affiliations of authors and, for English journals, the continents, countries or regions to which the authors belong; whereas for Chinese language journals, the provinces. Co-cited authors means that the authors have been cited together. VOSviewer (version 1.6.14) software was utilized to analyze the relationships among the most highly productive countries, research institutions, and frequently used keywords. We performed cluster analysis and generated social network maps (consist of nodes and links) for countries, institutions and keywords by VOSviewer (16, 17). Cluster was also obtained by VOSviewer via analyzing the frequency of the same keywords appearing within the different papers. We set either twice or four times as the

TABLE 1 | The top 10 authors and journals of COVID-19 research in English and Chinese [*n* (%)].

Rank	Authors in English	*N* (1,062, %)	Authors in Chinese	*N* (3,243, %)	Journals in English	*N* (143, %)	Journals in Chinese	*N* (721, %)
1	Li Y (Wuhan Uni)	7 (4.9)	Wang Y (Beijing Hosp TCM)	6 (0.8)	J Med Virol	18 (12.6)	Chin General Practice Nursing	41 (5.7)
2	Benvenuto D (Uni Campus Bio-medico of Rome)	5 (3.5)	Yang F (Tianjin Uni TCM)	6 (0.8)	Euro Surveillance	16 (11.2)	J Trad Chin Med	23 (3.2)
3	Eurosurveillance Editorial Team (European CDC)	5 (3.5)	Wang Y (Longhua Hosp, Shanghai Uni TCM)	5 (0.7)	Lancet	13 (9.1)	Chin Herb Med	18 (2.5)
4	Leung G (Uni Hong Kong)	5 (3.5)	Guo Y (1st Affiliated Hosp, Xi'an Jiaotong Uni)	4 (0.6)	Emerging Microbes Infect	6 (4.2)	World J Trad Chin Med	16 (2.2)
5	Angeletti S (Univ Campus Bio-medico of Rome)	4 (2.8)	Lei X (2nd Affiliated Hosp, Xi'an Jiaotong Uni)	4 (0.6)	NEJM.	6 (4.2)	Herald Med	15 (2.1)
6	Gao G (China CDC)	4 (2.8)	Li S (2nd Affiliated Hosp, Xi'an Jiaotong Uni)	4 (0.6)	Viruses	5 (3.5)	Shanghai Med J	14 (1.9)
7	Ran J (Uni Hong Kong)	4 (2.8)	Luo F (Union West China Hosp, Sichuan Uni)	4 (0.6)	Radiology	4 (2.8)	Chin J Tuberc Resp Dis	13 (1.8)
8	Wei Y (Wuhan Jinyintan Hosp)	4 (2.8)	Miao Q (Xiyuan Hosp, Academy Chinese Med Sci)	4 (0.6)	World J Pediatric	4 (2.8)	Chin J Prev Med	12 (1.7)
9	Wu J (Uni HongKong)	4 (2.8)	Shi J (Longhua Hosp, Shanghai Uni TCM	4 (0.6)	BML	3 (2.1)	Chongqing Med	11 (1.5)
10	Yang G (Chinese Uni Hong Kong)	4 (2.8)	Shu B (Longhua Hosp, Shanghai Uni TCM)	4 (0.6)	JAMA	3 (2.1)	China Trop Med/ Chin J Resp Critical Care Med/Chin Nur Res	10 (1.4)

minimum frequency of keywords occurrence in English or Chinese publications, respectively, reflecting the number of included studies (143 or 721, respectively) and the consequent analysis results. Thus, the main reason for the different settings between English and Chinese is because there are more than double the number of keywords from the Chinese vs. the English language papers. Consequently, there would be too many clusters if the frequency of keywords were set as twice for the Chinese publications. Different nodes in a map represent elements including a country, institution, or keywords. The size of the nodes reflects the number of publications or frequency, the larger the node, the greater the number of publications or frequency (18). The links between nodes represent relationships of collaboration, co-occurrence, or co-citations. The color of nodes and lines represents different clusters (19). The parameters of VOSviewer were as follows: counting method (fractional counting) and "ignore documents with a large number of authors" (maximum number of authors per document is 25).

CiteSpace is scientific software that reveals the trends and dynamics in scientific literature as well as identifies key points in a given research field (18, 20). CiteSpace was therefore used to design the social network. In the current study, CiteSpace was used to identify co-occurrence maps of authors, keywords, institutions, countries or provinces and capture keywords.

RESULTS

A total of 864 original research articles and reviews were included, of which, 143 were retrieved from Embase and Scope in English and 721 from SinoMed, CNKI, VIP and Wanfang in Chinese.

Authors and Journals

A total of 1,062 authors have been identified in the 143 articles published in 62 English journals. The top 10 authors and journals are listed (**Table 1**). The top 10 authors have contributed 46 (32.1%) of the papers. Author Li Y has the highest number of published papers (7, 4.9%), followed by Benvenuto D, Eurosurveillance Editorial Team and Leung GM (5, 3.5%), and Angeletti S, Gao GF, Ran J, Wei Y, Wu JT, and Yang G (4, 2.8%). The top 10 English journals are responsible for the publication of 72 (50.3%) papers, of which, J Med Virol is the highest (18, 12.6%), followed by Euro Surveillance (16, 11.2%) and Lancet (13, 9.1%) (**Table 1**).

Meanwhile, 3,243 authors have been identified in the 721 articles published in 193 Chinese journals. The top 10 authors have contributed 45 (6.2%) of the papers. Authors Wang YG and Yang FW have published the highest number of papers (6, 0.8%), followed by Wang YJ (5, 6.9%). The top 10 Chinese journals have published 193 (26.8%) of the papers, the highest is Chinese

General Practice Nursing (41, 5.7%), followed by J Traditional Chin Med (23, 3.2%) and Chin Herb Med (18, 2.5%) (**Table 1**).

For the analysis of the social relationships of authors (affiliated institutions) with more than three articles (**Figure 1**), it was found that of 38 authors who published English papers, seven clusters corresponding to seven categories were identified (A), and of 29 authors who published Chinese papers, clustering identified eight categories (B). These categories demonstrate that the cooperation between the various authors is close.

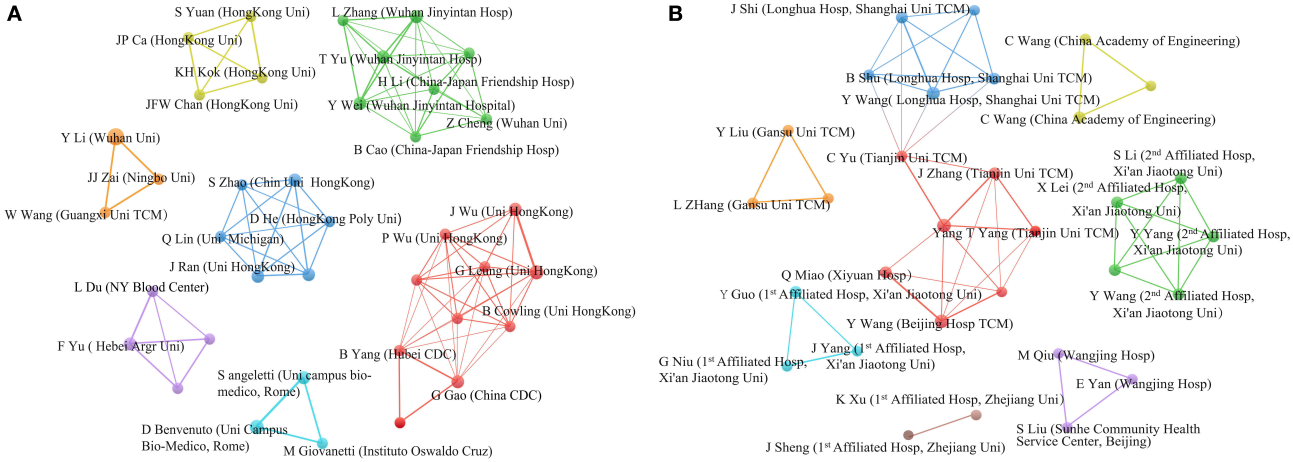


FIGURE 1 | The level of cooperation among the identified authors. **(A)** for English papers; **(B)** for Chinese papers.

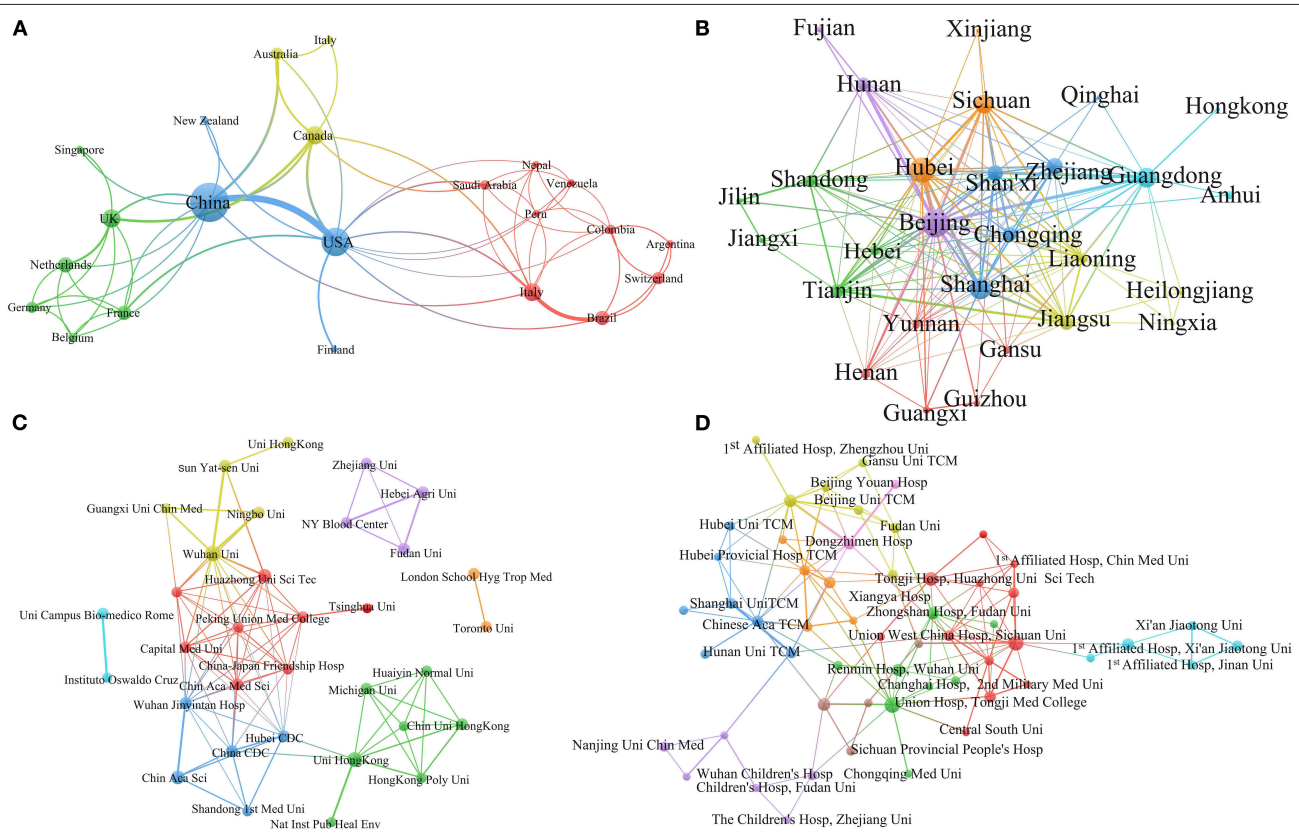


FIGURE 2 | (A) The cooperation among the countries **(A)** and provinces in China **(B)**; The cooperation among the institutions for English **(C)** and Chinese **(D)** publications.

Countries/Provinces (Areas) and Institutions

Of a total of 143 English papers that were published, there were a total of 1,062 authors from 32 countries or areas, including China (75/143, 52%), USA (34/143, 24%), UK (11/143, 8%), Canada (11/143, 8%), and Italy (10/143, 7%). There are 252 institutions from 32 countries published COVID-19 related English papers. The first five are from China, including Wuhan University (15/252, 6%), University of Hong Kong (12/252, 5%), Chinese Academy of Sciences (9/252, 4%), Huazhong University of Science and Technology (8/252, 3%), and Chinese CDC (6/252, 2%). For the analysis of the social relationships of countries with more than three articles (**Figure 2A**), 22/32 countries are clustered into four categories; 33/35 institutions are clustered into seven categories (**Figure 2B**), demonstrating the cooperation between countries and institutions is close.

Meanwhile, within the Chinese literature, of the 721 papers published there are 3,243 authors from 30 Provinces, autonomous regions or municipalities in China who contributed to the publications. The top five are Beijing (168, 23%), Hubei (136, 19%), Shanghai (90, 12%), Guangdong (78, 11%), and Sichuan (70, 10%). However, the provinces/Autonomous Regions who published the lowest number of articles in Chinese include Xinjiang Uygur, Qinghai and Ningxia, Inner Mongolia and Hong Kong, had 2 articles each. There are 677 institutions who published COVID-19 related papers. The institutions that have published the largest number of papers in Chinese are the Union Hospital, Huazhong University of Science & Technology (37, 5%), West China Union Hospital, Sichuan University (32, 5%), Tongji Hospital, Huazhong University of Science & Technology (23, 3%), Beijing University of Chinese Medicine (18, 3%), Zhongnan Hospital, Wuhan University (16, 2%) (**Table 2**).

For the analysis of the social relationships of provinces/areas with more than three articles, as can be seen from **Figure 2C**, amongst 32 provinces/areas, 28 provinces/areas are clustered into seven categories; amongst 677 institutions, 56 are clustered into nine categories, and the cooperation between them is close with more than three articles (**Figure 2D**).

TABLE 2 | The top five countries/areas and institutions that contributed to publications of COVID-19 research in English and Chinese [*n* (%)].

Rank	Countries/areas	*N* (%)	Institution	*N* (%)
COUNTRIES IN ENGLISH JOURNALS				
1	China	75 (52)	Wuhan University, Hubei	15 (6)
2	USA	34 (24)	University of HongKong	12 (5)
3	UK	11 (8)	Chinese Academy of Sciences	9 (4)
4	Canada	11 (8)	Huazhong Uni Sci Technol, Hubei	8 (3)
5	Italy	10 (7)	Chinese CDC	6 (2)
PROVINCES IN CHINESE JOURNALS				
1	Beijing	168 (23)	Union Hosp, Tongji Med College, Huazhong Uni Sci Technol, Hubei	37 (5)
2	Hubei	136 (19)	Union West China Hosp, Sichuan Uni	32 (5)
3	Shanghai	90 (12)	Tongji Hosp, Tongji Med College, Huazhong Uni Sci Technol, Hubei	23 (3)
4	Guangdong	78 (11)	Beijing Uni Chin Med	18 (3)
5	Sichuan	70 (10)	Zhongnan Hosp, Wuhan Uni, Hubei	16 (2)

Co-occurrence of Keywords

For the papers published in English, 471 English keywords are extracted from the 143 articles. A density map is generated for keywords with a co-occurrence greater than twice, including 54 keywords in the map (**Figure 3A**). SARS-CoV-2 was the most frequently used keyword (**Figure 3A**), with 93 (19.7%) co-occurrences, followed by COVID-19 (44, 9.3%), China (36, 7.6%), SARS (22, 4.8%), and epidemic (17, 3.6%) (**Table 3**). Among the top 20 keywords, some are related to epidemiological characteristics, such as epidemic, adult, male, female, travel, others are related to a comparison with similar diseases, e.g., MERS, SARS. Some are correlated to the structure of the virus, e.g., endogenous compound, amino acid, cladistics, and phylogeny. Cluster analysis is performed on co-occurrence of English keywords with a frequency >2. There are 54 keywords clustered into five categories (**Supplementary Figure 1B**). Cluster 1 includes 22 keywords, adult, clinical feature, clinical laboratory, coughing, diarrhea, female, fever, gene expression, high throughput sequence, Hong Kong, intensive care unit, lymphocytopenia, male, mortality, pneumonia, protein expression, sea food, thorax radiography, travel, virus pneumonia and World Health Organization; Cluster 2 mainly focuses on China, coronavirus, epidemic, influenza virus, phylogenetic tree, public health, reverse transcription polymerase chain reaction, virus detection, virus genome, virus transmission; Cluster 3 mainly focuses on COVID-19, drug, emerging virus, genome, MERS, outbreak, SARS, virus and Wuhan.

A total of 1,234 Chinese keywords are extracted from the 721 Chinese-language articles. A density map is generated for keywords with a co-occurrence >4 times, resulting in the generation of five categories (**Table 3**). As stated above, there are substantial more Chinese keywords identified within the Chinese Journals. If thence-occurrence of three times or less is adopted for the analysis, the clusters would be too many to offer an objective outcome. COVID-19 is the most frequently used keyword, with 543 (44.0%) co-occurrence (**Figure 3B**), followed by SARS-CoV-2 (381, 30.9%), TCM (153, 12.4%), prevention and control (141, 11.4%), epidemic (56, 4.5%), management (51, 4.1%), therapeutics (48, 3.9%), and computed tomography (CT) (35, 2.8%). Among the selected top 30 keywords with frequency more than 10, there were five clusters generated with such information (**Figure 3B**). For more detailed clusters, these were as follows: The keywords from the cluster one included clinical symptoms, critical case, CT, diagnosis, nucleic acids, therapeutics, X-ray; the keywords from the cluster two included cancer patients, emergency, infection, management, medical care personnel, mental health, prevention and control; the keywords for the

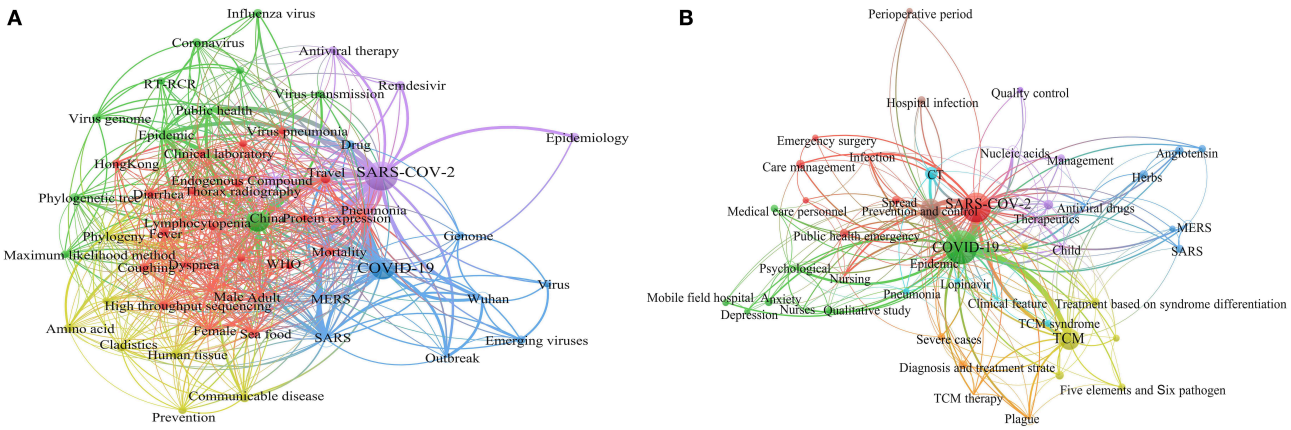


FIGURE 3 | The cluster analysis of English **(A)** and Chinese **(B)** keywords.

TABLE 3 | The top 20 keywords in terms of frequency for COVID-19 research in English and Chinese [*n* (%)].

Rank	English keywords	N (471, %)	Chinese keywords	*N* (1,234, %)
1	SARS-CoV-2	93 (19.7)	COVID-19	543 (44.0)
2	COVID-19	44 (9.3)	SARS-CoV-2	381 (30.9)
3	China	36 (7.6)	Prevention and control	141 (11.4)
4	SARS	22 (4.8)	Traditional Chinese Medicine	140 (11.3)
5	Epidemic	17 (3.6)	Computed tomography	35 (2.8)
6	Adult	15 (3.2)	Epidemic	29 (2.4)
7	Psychological	15 (3.2)	Public health	24 (1.9)
8	Nucleic acids	12 (2.5)	MERS	21 (1.7)
9	Plague	11 (2.3)	Pneumonia	21 (1.7)
10	Infection	11 (2.3)	Male	20 (1.6)
11	Child	8 (1.7)	Wuhan	19 (1.6)
12	Antiviral drugs	8 (1.7)	Endogenous compound	18 (1.5)
13	Nursing	8 (1.7)	Female	18 (1.5)
14	Therapeutics	6 (1.3)	Phylogeny	15 (1.5)
15	Diagnosis	5 (1.1)	Outbreak	14 (1.2)
16	MERS	5 (1.1)	Communicable disease	13 (1.1)
17	Clinical feature	5 (1.1)	Fever	13 (1.1)
18	Nurses	4 (0.9)	Travel	13 (1.1)
19	Amino acid	4 (0.9)	Traditional Chinese Medicine therapy	13 (1.1)
20	Angiotensin	4 (0.9)	Cladistics	9 (0.9)

cluster three were Covid-19, epidemic, guidelines, integrated Chinese & Western medicine, TCM, the scheme of diagnosis and treatment, treatment based on syndrome differentiation; the cluster four included angiotensin, antiviral drugs, herbs, MERS, SARS-COV-2; finally the cluster five were nursing and pregnant.

Network Social Analysis Between China and Other Countries

There are total 143 papers written in English within this study from which data has been collected. Of these 143 papers, 21 articles were written jointly between Chinese authors and authors from other countries, while 54 papers published in English were authored by Chinese authors only. Among the 21 articles authored jointly, the order of co-operations was: China and USA (14/21), China and UK (3/21), and then Australia and Canada (2/21). To demonstrate this point, a network social analysis was performed (**Figure 2A**).

For the 21 English language articles jointly authored between Chinese and international authors, the institutions involved in cooperation between China and other countries were found to be centered in Hong Kong, Hubei Province, Beijing and Shanghai within China, while the most frequent overseas institution involved in cooperation was the New York Blood Center from the USA (**Figure 2C**). This cooperation covered a range of scientific topics, mainly focusing on diagnosis, such as PCR testing in the laboratory, prevention and control, and the viral genome (**Figure 3A**).

DISCUSSION

The battle against COVID-19 has been highly effective in China up to date, however, the pandemic of COVID-19 is highly alarming in around the world with substantial morbidity and mortality (3). The most urgent task for medical doctors/scientists is to control COVID-19, including the incorporation of aspects of the Chinese approaches. Many diverse studies addressing COVID-19 have sprung up due to the urgent necessity of prevention and control.

We have focused on English and Chinese publications only for the comparison. Most of the studies captured in this paper on COVID-19 in English journals have been conducted by Chinese scholars and institutions, which is highly likely to be due to the timing of the literature search for this study, March 1st 2020, at which point the predominantly affected locations were Wuhan, and to a lesser extent the remainder of China. Of the international publications, particularly from Western countries, e.g., Italy (21) and South Korea (22), these publications

occurred during the latter part of the survey period, from 27 February 2020, which is likely to be attributed to the spread of the SARS-CoV-2 commencing within these other countries, both raising the index of concern within those other regions and directly making available to those regions affected local populations and biological materials on which studies could be conducted. More authors from Hong Kong published more English papers than papers in Chinese, which may be due to the higher levels of advanced English literacy, reflecting the English-based educational system (23). Furthermore, the majority of the Hong Kong researchers have more opportunity to study/work and establish links overseas (24), in addition to their preference for English journals. Although the impact of COVID-19 in Iran has been very severe (25), there has been no studies published on the pandemic at all prior to March 1st, 2020. We speculate that the Iranian government has experienced difficulties scaling up its response to combating the epidemic, due to the economic loss and supply issues associated with economic sanctions imposed (26).

Similarly, the scholars who published studies on COVID-19 in Chinese journals are mainly from Beijing, Hubei, Shanghai, Guangdong and Sichuan. A likely explanation is that most of the first-class medical universities are within these areas, corresponding to the top research institutional distribution in China. Apart from Wuhan, Hubei Province, Sichuan University has published more papers than other areas, except for Beijing, Shanghai and Guangdong, which are the three provinces with the highest GDP in China (27). Especially relevantly, as the capital of China, Beijing is the nation's political, economic, cultural and educational center, and has the largest number of universities in the country. These data support the idea that advanced academic development needs financial support. Certainly, less publications are from Xinjiang Uygur, Qinghai, Ningxia, and Inner Mongolia, all of which has fewer COVID-19 cases, but also have lower GDP within remote northwest China (GDP rankings out of 31 regions 19, 23, 15, 9, respectively) (28). In the cluster of authors, we found that the cooperation between the various authors is close but there is not a hotspot amongst them, which is in line with the reality that the information sharing was lacking at the early stage.

The studies in English related to COVID-19 are published in international, highly reputable journals, including Nature, Science, Cell, NEJM, JAMA, and Lancet. These publications enable medical practitioners/scientists to share/exchange information efficiently, providing essential background for some key policy decisions (29, 30), e.g., mandatory wearing of face masks, minimizing social gathering [has been widely accepted, including Australia (31), UK (32)], and the lockdown of interstate travel in many countries of the EU (33). Importantly, the ultra-rapid development of an effective vaccine, has been accelerated by the rapid sharing of scientific data, particularly the published sequences of the SARS-CoV-2 virus. Thus, publications in English journals, particularly in well-recognized, top ranking international journals, results in rapid dissemination of key information for use of the data for practical applications. English is the well-accepted communication language of science around the world.

Our data demonstrated that substantial collaborative research has been undertaken from the very early period of the COVID-19 outbreak (34, 35), and that this research has become more frequent and deep following the declaration of a pandemic. Such collaborations are certainly enhancing our understanding of the nature of the SARS-CoV-2 virus (36, 37), have supported development of effective vaccines (38, 39), and has provided vital data to assist clinical diagnosis at the international level (40). These developments further support our conclusion that publications in English have enabled doctors/scientists to effectively share/exchange information at the international level. The cluster analysis of institutions at the international level demonstrated strong regional representation even at the international level, both within China and within international countries. Interestingly, the cluster of cooperation for studies in China was thickest with the USA, suggesting the cooperation was mainly between China and USA, which is consistent with the publications retrieved from the database. The most likely explanation has been mentioned above, namely that the economic resources of each country is the likely most significant factor to impact both the disease and research into it.

In contrast, there is a language barrier to the utilization of the information from the papers published in English journals for use by the general population in China. The publications in the Chinese language are able to meet a complementary dissemination purpose for China-based medical practitioners to understand the essential key information concerning COVID-19, especially for those in the remote areas of China, without proper access of English journals or sufficient language skills (41). Indeed, publications in Chinese provide a more acceptable approach for Chinese doctors to learn how to deal with COVID-19 in the relatively remote regions of China, an outcome that is consistent with the large number of studies that have been published in Chinese.

The top 10 Chinese journals that included COVID-19-related papers are mostly from the Chinese Science Citation Database, representing the most authoritative and representative core of journals in all disciplines in China (42). Importantly, in this study there are a total of 721 papers that cover COVID-19 from various scientific areas within the identified journals, often with a large number of authors, reflecting the Chinese authority's intention to accelerate the control of COVID-19 and the rapid dissemination of knowledge.

There are a total 471 or 1,234 keywords in English or in Chinese publications, respectively, used in the studies on COVID-19 that we identified till March 1st, 2020. However, more than 78% of the keywords appeared once, only 3.9% of the English keywords have a frequency of >4, indicating the importance of a few keywords. In bibliometrics, a network graph of keyword co-occurrences reflects hot topics (18). Cluster analysis of co-occurrence keywords demonstrates that there are five clusters in this field. Cluster 1 consists of 22 keywords, mainly relates to the epidemiological characteristics and clinical features, because these are the basis for understanding key aspects of the disease, such as treatment and control. At the present time, many scholars are focusing on the large proportion of COVID-19 patients who exhibit mild symptoms

or are asymptomatic carriers, reflecting the seriousness of the nature of viral transmission (43). Cluster 2 contains 11 keywords, mainly focuses on the virus detection and genome. Some data demonstrate that bat CoV and human SARS-CoV-2 might share the same ancestor (40), and similar residues of the key receptor are observed in many species (44). Because of the importance of the original source of SARS-CoV-2, the evolution and genomics is a hot topic in this field. Nine keywords are included in cluster 3, focusing on drug treatment and comparison with SARS and MERS, making comparisons to these fatal respiratory tract infections by coronaviridae, to explore any clues between the similarity and differentiation.

For the papers published in Chinese language journals, there are five clusters of keywords, including 2–6 keywords in each field. Cluster 1 consists of six keywords, mainly relating to treatment and diagnosis, because these activities are the basis for understanding key aspects of the disease, such as treatment and control. At the present time, many scholars are focusing on the large proportion of COVID-19 patients who exhibit mild symptoms or are asymptomatic carriers, reflecting the seriousness of the nature of viral transmission (43). Cluster 2, contains 6 keywords, mainly focusing on emergency, infection, management, medical carers, prevention and control, which are supported by the others, demonstrating the critically importance of COVID-19 in such outbreak (45), transmission (46) and disease control and management (47). Cluster 3 is focusing on TCM or Chinese and Western treatment for COVID-19, mainly to explore the benefit of the combination of TCM and classical Western management approaches, especially aiming to provide the guidelines for relatively remote/rural regions of China. The advantage of this particular cluster is its usefulness in the outskirts of metropolitan or rural areas, where there is a relative lack of advanced or first line anti-viral medications (48). Cluster 4 is an extension of the current existing treatment to the cardiovascular system (49), as well as, using the previous experience in MERS (50), and also places emphasis on anti-viral drugs and herbs (51). Interestingly, cluster 5 includes pregnancy and nursing, which is a very venerable population at high risk, either due to compromised immunity during pregnancy (52) or the lack of sufficient data to adequately understand the severity of the potential risk of COVID-19 in pregnancy and the need to guard against COVID-19 infection in pregnancy (53).

Part of the reason for the Chinese scholars focus on TCM when publishing in Chinese medical journals is the difficulty Chinese scholars have to disseminate their findings using modern scientific terminology/theory, compared to rather ancient theory of TCM, e.g., balance of Ying and Yang. Actually, we believe that balance of Ying and Yang is equivalent to the modern theory of anti-vs. pro-inflammatory responses in the micro-environment, i.e., imbalance of anti-vs. pro-inflammatory responses contributes to autoimmune diseases (54). Thus, from the point of view of the management of COVID-19, the efforts should be focused on the suppression of the SARS-CoV-2 virus, disregarding the backgrounds, theories, and approaches of modern vs. traditional scientific ideology. Consequently, analysis of the dissemination of the critical information from English and Chinese languages could facilitate such a purpose appropriately.

These COVID-19 related English language papers, especially at the top end, e.g., NEJM (55), Lancet (7, 26), Science (8), Nature (36), provide the most critical information in the development of effective vaccinations (31). On the other hand, for many primary health carers at the front line in the relatively remote regions in China, obtaining the most up dated information of COVID-19 particularly, regarding prevention and/or controlling has been from the Chinese language. In addition, the local government at the county levels are also heavily dependent on such key information in Chinese, in detail, e.g., keep social distance, no public gathering, and lockdown of manufacture and so on (56).

There are some limitations in the current study. First, our study is focusing only on English and Chinese journals, which inevitably could miss some important information from other languages. We will further analyze such points by collaborating with researchers from the different regions/countries. Second, our study has been undertaken at the vortex of the epidemic before March, 2020, which may miss the most updated information. Third, the total number of included studies is relatively small, and the study duration of just more than 2 months from when the first COVID-19 patient is identified till March 1st, 2020, is a short cutoff time for data retrieval.

CONCLUSIONS

The publications related to COVID-19 research has been rapidly growing since the disease emerged. More studies have been published in Chinese journals than in English, due to the epicenter being located in Wuhan, China before March 1st 2020. The publications in English have enabled doctors/scientists to share/exchange information at the international level; the publications in the Chinese language provides complementary educational approaches for the local doctors to understand the essential and key information to manage COVID-19 in the relatively remote regions of China for the general population.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

JF and SB conceived of the presented idea. JF and YG developed the theory and performed the computations. YG and JT verified the analytical methods. NZ, RD, HZ, XF, and GS collected and synthesized the data. CC and BH encouraged JF and YG to investigate and supervised the findings of this

work. All authors discussed the results and contributed to the final manuscript.

FUNDING

This work was supported by the Talent Introduction Program of Gansu University of Chinese Medicine [grant number 2016YJRC-01]; the Innovation Capacity Improvement Special Project on COVID-19 Prevention and Control from Gansu University of Chinese Medicine [grant number 2020XGZX-10]; University Innovation Capacity Improvement Project in Gansu Province; and SJTU grants [grant number 2019].

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2020.00477/full#supplementary-material

Supplementary Figure 1 | The density map of English **(A)** and Chinese **(B)** keywords for COVID-19 publications.

REFERENCES

1. World Health Organization. *WHO Director-General's Opening Remarks at the Media Briefing on COVID-19 - 5 March 2020*. (2020). Available online at: https://www.who.int/dg/speeches/detail/who-director-general-s-opening-remarks-at-the-media-briefing-on-covid-19---5-march-2020 (accessed March 5, 2020).
2. Chen T, Rui J, Wang Q, Zhao Z, Cui J, Yin L. A mathematical model for simulating the phase-based transmissibility of a novel coronavirus. *Infect Dis Poverty*. (2020) 9:24. doi: 10.1186/s40249-020-00640-3
3. World Health Organization. (2020). Available online at: https://www.who.int/docs/default-source/coronaviruse/situation-reports/20200627-covid-19-sitrep-159.pdf?sfvrsn=93e027f6_2 (accessed June 28, 2020).
4. National Health Commission of The People's Republic of China. *Prevention and control plan of COVID-19 (The Seventh Edition)*. (2020). Available online at: http://www.nhc.gov.cn/xcs/fkdt/202002/dc7f3a7326e249c0bad0155960094b0b.shtml (accessed February 21, 2020).
5. Ministry of Education, The People's Republic of China. *MOE Party Leadership Group Issues Notice for COVID-2019 Control and Educational Reforms*, (2020). Available online at: http://en.moe.gov.cn/news/press_releases/202003/t20200302_426475.html (accessed March 2, 2020).
6. National Health Commission of The People's Republic of China. *Situation Report-Update on COVID-19 Up to July 31, 2020*. (2020). Available online at: http://www.nhc.gov.cn/xcs/yqfkdt/202008/96184996b6724df787ea9380dfdc88bf.shtml (accessed August 1, 2020).
7. Xu Z, Shi L, Wang Y, Zhang J, Huang L, Zhang C, et al. Pathological findings of COVID-19 associated with acute respiratory distress syndrome. *Lancet Respir Med*. (2020) 4:420–2. doi: 10.1016/S2213-2600(20)30076-X
8. Wrapp D, Wang N, Corbett KS, Goldsmith JA, Hsieh CL, Abiona O, et al. Cryo-EM structure of the 2019-nCoV spike in the prefusion conformation. *Science*. (2020) 367:1260–3. doi: 10.1126/science.abb2507
9. Li L, Liang X, Jiang Q, Wang H, Wang B. An update on the epidemiological characteristics of novel coronavirus pneumonia COVID-19. *Chin J Epidemiol*. (2020) 41:139–44. doi: 10.3760/cma.j.issn.0254-6450.2020.02.002
10. Xiang T, Liu J, Xu F, Cheng N, Liu Y, Qian K, et al. Analysis of clinical characteristics of 49 patients with Novel Coronavirus Pneumonia in Jiangxi province. *Chin J Resp Critical Care Med*. (2020) 19:1–17.
11. Zhang M, Gao M, Yue S, Zheng T, Gao Z, Ma X, et al. Global trends and future prospects of food waste research: a bibliometric analysis. *Environ Sci Pollut Res Int*. (2018) 25:24600–10. doi: 10.1007/s11356-018-2598-6
12. Chahrour M, Assi S, Bejjani M, Nasrallah AA, Salhab H, Fares M, et al. A bibliometric analysis of COVID-19 research activity: a call for increased output. *Cureus*. (2020) 12:e7357. doi: 10.7759/cureus.7357
13. Lou J, Tian SJ, Niu SM, Kang XQ, Lian HX, Zhang LX, et al. Coronavirus disease 2019: a bibliometric analysis and review. *Eur Rev Med Pharmacol Sci*. (2020) 24:3411–21. doi: 10.26355/eurrev_202003_20712
14. Felice FDE, Polimeni A. Coronavirus Disease (COVID-19): A Machine learning bibliometric analysis. *In vivo*. (2020) 34(3 Suppl.):1613–7. doi: 10.21873/invivo.11951
15. Sun J, Guo Y, Scarlat MM, Lv G, Yang XG, Hu YC. Bibliometric study of the orthopaedic publications from China. *Int Orthop*. (2018) 42:461–8. doi: 10.1007/s00264-018-3828-8
16. Mulet-Forteza C, Genovart-Balaguer J, Merigó JM, Mauleon-Mendez E. Bibliometric structure of *IJCHM* in its 30 years. *IJCHM*. (2019) 12:4574–604. doi: 10.1108/IJCHM-10-2018-0828
17. Merigó JM, Mulet-Forteza C, Valencia C, Lew AA. Twenty years of *Tourism Geographies*: a bibliometric overview. *Tourism Geographies*. (2019) 21:881–910. doi: 10.1080/14616688.2019.1666913
18. Liang Y, Li Y, Zhao J, Wang X, Zhu H, Chen X. Study of acupuncture for low back pain in recent 20 years: a bibliometric analysis via CiteSpace. *J Pain Res*. (2017) 10:951–64. doi: 10.2147/JPR.S132808
19. Gao Y, Ge L, Shi S, Sun Y, Liu M, Wang B, et al. Global trends and future prospects of e-waste research: a bibliometric analysis. *Environ Sci Pollut Res Int*. (2019) 26:17809–20. doi: 10.1007/s11356-019-05071-8
20. Li C, Wu K, Wu J. A bibliometric analysis of research on haze during 2000-2016. *Environ Sci Pollut Res Int*. (2017) 24:24733–42. doi: 10.1007/s11356-017-0440-1
21. Livingston E, Bucher K. Coronavirus Disease 2019 (COVID-19) in Italy. *JAMA*. (2020) 14:1335. doi: 10.1001/jama.2020.4344
22. Shim E, Tariq A, Choi W, Lee Y, Chowell G. Transmission potential and severity of COVID-19 in South Korea. *Int J Infect Dis*. (2020) 93:339–44. doi: 10.1016/j.ijid.2020.03.031
23. Education Bureau. *The Government of the Hong Kong Special Administrative Region*. (2019). Hong Kong Education System. Available online at: https://www.studyinhongkong.edu.hk/en/hong-kong-education/education-system.php (accessed 2019).
24. *The Educaiton Abroad Network. Hong Kong Internship*. Available online at: https://teanabroad.org/programs/hong-kong-internship/ (accessed January 29, 2020).
25. Arab-Mazar Z, Sah R, Rabaan A, Dhama K, Rodriguez-Morales AJ. Mapping the incidence of the COVID-19 hotspot in Iran - Implications for Travellers. *Travel Med Infect Dis*. (2020) 34:101630. doi: 10.1016/j.tmaid.2020.101630
26. Takian A, Raoofi A, Kazempour-Ardebili S. COVID-19 battle during the toughest sanctions against Iran. *Lancet*. (2020) 395:1035–6. doi: 10.1016/S0140-6736(20)30668-1
27. National Bureau of Statistics of China. Annual Data – GDP (2020). Available online at: http://data.stats.gov.cn/search.htm?s=GDP (accessed January 16, 2020).
28. Wikipedia. *List of Chinese Administrative Division by GDP Per Capita*. (2019). Available online at: https://en.wikipedia.org/wiki/List_of_Chinese_administrative_divisions_by_GDP_per_capita (accessed April 16, 2019).
29. Fan J, Liu X, Pan W, Douglas MW, Bao S. Epidemiology of 2019 novel coronavirus disease-19 in gansu province, China, 2020. *Emerg Infect Dis*. (2020) 6:1257–65. doi: 10.3201/eid2606.200251
30. World Health Organization. *Advice on the Use of Masks in the Community, During Home Care and in Health Care Settings in the Context of the Novel Coronavirus (2019-nCoV) Outbreak: Interim Guidance*. (2020). Available online at: https://www.who.int/docs/default-source/coronaviruse/advice-on-the-use-of-masks-2019-ncov.pdf (accessed January 29, 2020).
31. Department of Health Australia Government. *Limits on Public Gatherings for Coronavirus (COVID-19)*. (2020). Available online at: https://www.health.gov.au/news/health-alerts/novel-coronavirus-2019-ncov-health-alert/how-to-protect-yourself-and-others-from-coronavirus-covid-19/limits-on-public-gatherings-for-coronavirus-covid-19 (accessed March 30, 2020).

32. UK Government. *COVID-19 Guidance for Mass Gatherings*. (2020). Available online at: https://www.gov.uk/guidance/covid-19-guidance-for-mass-gatherings (accessed March 16, 2020).
33. United Nations in Western Europe. *COVID-19: EU Suspends Non-Essential Travel for Non-EU Citizens*. (2020). Available online at: https://unric.org/en/covid-19-european-union-suspends-non-essential-travel-for-non-eu-citizens/ (accessed March 17, 2020).
34. Lu R, Zhao X, Li J, Niu P, Yang B, Wu H, et al. Genomic characterisation and epidemiology of 2019 novel coronavirus: implications for virus origins and receptor binding. *Lancet.* (2020) 395:565–74. doi: 10.1016/S0140-6736(20)30251-8
35. Zhao S, Lin Q, Ran J, Musa SS, Yang G, Wang W, et al. Preliminary estimation of the basic reproduction number of novel coronavirus (2019-nCoV) in China, from 2019 to 2020: a data-driven analysis in the early phase of the outbreak. *Int J Infect Dis.* (2020) 92:214–7. doi: 10.1101/2020.01.23.916395
36. Jin Z, Du X, Xu Y, Deng Y, Liu M, Zhao Y, et al. Structure of M(pro) from SARS-CoV-2 and discovery of its inhibitors. *Nature.* (2020) 582:289–93. doi: 10.1038/s41586-020-2223-y
37. Shang J, Ye G, Shi K, Wan Y, Luo C, Aihara H, et al. Structural basis of receptor recognition by SARS-CoV-2. *Nature.* (2020) 581:221–4. doi: 10.1038/s41586-020-2179-y
38. Tai W, He L, Zhang X, Pu J, Voronin D, Jiang S, et al. Characterization of the receptor-binding domain (RBD) of 2019 novel coronavirus: implication for development of RBD protein as a viral attachment inhibitor and vaccine. *Cell Mol Immunol.* (2020) 17:613–20. doi: 10.1038/s41423-020-0400-4
39. Smith TRF, Patel A, Ramos S, Elwood D, Zhu X, Yan J, et al. Immunogenicity of a DNA vaccine candidate for COVID-19. *Nat Commun*. (2020) 11:2601. doi: 10.1038/s41467-020-16505-0
40. Corman VM, Landt O, Kaiser M, Molenkamp R, Meijer A, Chu DK, et al. Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. *Euro Surveill.* (2020) 25:2000045. doi: 10.2807/1560-7917.ES.2020.25.3.2000045
41. Tang X, Wang Q. Research and reflections on english competence of medical professionals based on needs analysis. *China Educ Technol Equipment.* (2017) 3:109–11.
42. National Science Library, Chinese Academy of Sciences. *Introduction of Chinese Science Citation Database*. (2014). Available online at: http://sciencechina.cn/index_more1.jsp (accessed May 31, 2020).
43. Qiu J. Covert coronavirus infections could be seeding new outbreaks. *Nature.* (2020). doi: 10.1038/d41586-020-00822-x. [Epub ahead of print].
44. Zhou P, Yang X, Wang X, Hu B, Zhang L, Zhang W, et al. A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature.* (2020) 579:270–3. doi: 10.1038/s41586-020-2012-7
45. Sriwijitalai W, Wiwanitkit V. treatment during COVID-19 outbreak. *J Biol Dermatol Treat.* (2020) 31:324. doi: 10.1080/09546634.2020.1749548
46. Schwartz KL, Murti M, Finkelstein M, Leis JA, Fitzgerald-Husek A, Bourns L, et al. Lack of COVID-19 transmission on an international flight. *CMAJ.* (2020) 192:E410. doi: 10.1503/cmaj.75015
47. Willan J, King AJ, Jeffery K, Bienz N. Challenges for NHS hospitals during COVID-19epidemic. *BMJ.* (2020) 368:m1117. doi: 10.1136/bmj.m1117
48. Ni L, Zhou L, Zhou M, Zhao J, Wang DW. Combination of western medicine and Chinese traditional patent medicine in treating a family case of COVID-19. *Front Med.* (2020) 14:210–4. doi: 10.1007/s11684-020-0757-x
49. Zheng YY, Ma YT, Zhang JY, Xie X. COVID-19 and the cardiovascular system. *Nat Rev Cardiol.* (2020) 17:259–60. doi: 10.1038/s41569-020-0360-5
50. Rockx B, Kuiken T, Herfst S, Bestebroer T, Lamers MM, Oude Munnink BB, et al. Comparative pathogenesis of COVID-19, MERS, and SARS in a nonhuman primate model. *Science.* (2020) 368:1012–5. doi: 10.1126/science.abb7314
51. Mitjà O, Clotet B. Use of antiviral drugs to reduce COVID-19 transmission. *Lancet Glob Health.* (2020) 8:e639–e640. doi: 10.1016/S2214-109X(20)30114-5
52. Ling C. Traditional Chinese medicine is a resource for drug discovery against 2019 novel coronavirus (SARS-CoV-2). *J Integr Med.* (2020) 18:87–8. doi: 10.1016/j.joim.2020.02.004
53. Zhang Q, Wang Y, Qi C, Shen L, Li J. Clinical trial analysis of 2019-ncov therapy registered in China. *J Med Virol.* (2020) 92:540–5. doi: 10.1002/jmv.25733
54. Park JH, Lee KH, Jeon B, Ochs HD, Lee JS, Gee HY, et al. Immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome: a systematic review. *Autoimmun Rev*. (2020) 19:102526. doi: 10.1016/j.autrev.2020.102526
55. Grein J, Ohmagari N, Shin D, Diaz G, Asperges E, Castagna A, et al. Compassionate Use of Remdesivir for Patients with Severe COVID-19. *N Engl J Med.* (2020) 382:2327–36. doi: 10.1056/NEJMc2015312
56. Lio CF, Cheong HH, Lei CI, Lo IL, Yao L, Lam C, et al. The common personal behavior and preventive measures among 42 uninfected travelers from the Hubei Province, China during COVID-19 outbreak: a cross-sectional survey in Macao SAR, China. *PeerJ.* (2020) 8:e9428. doi: 10.7717/peerj.9428

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Medicine

ORIGINAL RESEARCH
published: 14 August 2020
doi: 10.3389/fmed.2020.00518

Performance of Two Risk-Stratification Models in Hospitalized Patients With Coronavirus Disease

Rong Xu[1†], Keke Hou[2†], Kun Zhang[1†], Huayan Xu[1], Na Zhang[2], Hang Fu[1], Linjun Xie[1], Ran Sun[1], Lingyi Wen[1], Hui Liu[1], Zhigang Yang[3], Ming Yang[4] and Yingkun Guo[1*‡]*

[1] Key Laboratory of Birth Defects and Related Diseases of Women and Children of Ministry of Education, Department of Radiology, West China Second University Hospital, Sichuan University, Chengdu, China, [2] Department of Radiology, Public Health Clinical Center of Chengdu, Chengdu, China, [3] Department of Radiology, West China Hospital, Sichuan University, Chengdu, China, [4] Department of Respiratory Medicine, Public Health Clinical Center of Chengdu, Chengdu, China

Background: Despite an increase in the familiarity of the medical community with the epidemiological and clinical characteristics of coronavirus disease 2019 (COVID-19), there is presently a lack of rapid and effective risk stratification indicators to predict the poor clinical outcomes of COVID-19 especially in severe patients.

Methods: In this retrospective single-center study, we included 117 cases confirmed with COVID-19. The clinical, laboratory, and imaging features were collected and analyzed during admission. The Multi-lobular infiltration, hypo-Lymphocytosis, Bacterial coinfection, Smoking history, hyper-Tension and Age (MuLBSTA) Score and Confusion, Urea, Respiratory rate, Blood pressure, Age 65 (CURB65) score were used to assess the death and intensive care unit (ICU) risks in all patients.

Results: Among of all 117 hospitalized patients, 21 (17.9%) patients were admitted to the ICU care, and 5 (4.3%) patients were died. The median hospital stay was 12 (10–15) days. There were 18 patients with MuLBSTA score $\geq$ 12 points and were all of severe type. In severe type, ICU care and death patients, the proportion with MuLBSTA $\geq$ 12 points were greater than that of CURB65 score $\geq$ 3 points (severe type patients, 50 vs. 27.8%; ICU care, 61.9 vs. 19.0%; death, 100 vs. 40%). For the MuLBSTA score, the ROC curve showed good efficiency of diagnosis death (area under the curve [AUC], 0.956; cutoff value, 12; specificity, 89.5%; sensitivity, 100%) and ICU care (AUC, 0.875; cutoff value, 11; specificity, 91.7%; sensitivity, 71.4%). The K–M survival analysis showed that patients with MuLBSTA score $\geq$ 12 had higher risk of ICU (log-rank, $P = 0.001$) and high risk of death (log-rank, $P = 0.000$).

Conclusions: The MuLBSTA score is valuable for risk stratification and could effectively screen high-risk patients at admission. The higher score at admission have higher risk of ICU care and death in patients infected with COVID.

Keywords: risk-stratification, coronavirus disease, MuLBSTA score, CURB65 score, ICU

OPEN ACCESS

Edited by:
Tamas Szakmany,
Cardiff University, United Kingdom

Reviewed by:
Natalija Vukovic,
University of Niš, Serbia
Eizo Watanabe,
Chiba University, Japan
Helio Penna Guimaraes,
Albert Einstein Israelite Hospital, Brazil

***Correspondence:**
Ming Yang
274453563@qq.com
Yingkun Guo
gykpanda@163.com

† These authors have contributed equally to this work

‡ORCID:
Yingkun Guo
orcid.org/0000-0001-8437-9887

Specialty section:
This article was submitted to Intensive Care Medicine and Anesthesiology, a section of the journal Frontiers in Medicine

Received: *14 May 2020*
Accepted: *27 July 2020*
Published: *14 August 2020*

Citation:
Xu R, Hou K, Zhang K, Xu H, Zhang N, Fu H, Xie L, Sun R, Wen L, Liu H, Yang Z, Yang M and Guo Y (2020) Performance of Two Risk-Stratification Models in Hospitalized Patients With Coronavirus Disease. Front. Med. 7:518. doi: 10.3389/fmed.2020.00518

INTRODUCTION

In December 2019, a series of pneumonia cases of unknown cause emerged in Wuhan, Hubei Province, China. Subsequently, a novel coronavirus was isolated and is known as the 2019 novel coronavirus (SARS-COV-2), which was designated as coronavirus disease 2019 (COVID-19) (1). With the worldwide prevalence and outbreak of COVID-19, the pressure regarding the prevention and treatment of this epidemic has intensified, and several local medical resources were seriously insufficient (2). Thus, understanding the risk stratification could help in the better allocation of the available medical resources as well as ensure appropriate clinical management of high-risk patients to improve the survival rate.

The clinical spectrum of COVID-19 infection appears to be wide, encompassing asymptomatic infection, mild upper respiratory tract illness, severe viral pneumonia with respiratory failure, shock, and even death. The current reported death rate is about 0.66–7.2% (1, 3–5). Some studies have published the risk factors that may be associated with poor prognosis, such as age or severe immune response (4–7). However, only a few studies focusing on clinical risk stratification, and the risk factors for in-hospital death or intensive care unit (ICU) care of patients were undefined.

An effective and comprehensive model for screening high-risk patients at admission is necessary for patients infected with SARS-COV-2. Therefore, we aimed to verify the efficacy of the Multi-lobular infiltration, hypo-Lymphocytosis, Bacterial coinfection, Smoking history, hyper-Tension, and Age (MuLBSTA) scale for mortality or ICU risk stratification in patients with COVID-19 and clarify the predictive value of the scale for poor prognosis.

METHODS

Study Design and Data Collection

We recruited patients from January 1 to March 25, 2020 in this retrospective study. All patients were diagnosed with COVID-19 pneumonia according to RT-PCR. All laboratory and imaging reports during the hospitalization were recorded. The institutional ethic committee of our institutes approved this study (No. 2020.43).

History of exposure, clinical manifestations, laboratory findings, CT characteristics, and epidemiological and outcome data were obtained from the collection forms and electronic medical records from admission to discharge. All recorded data were independently reviewed by two researchers.

CT Image Review

Signs and severity of lung lesions observed in Computed Tomography (CT) scans were evaluated, and lung involvement in each lobe was recorded. More than three lung lobes involvement were regarded as multi-lobular infiltrates. The "total severity score" was calculated by summing the five lobe scores (range: 0–25 points), and each of the five lung lobes were visually scored from 0 to 5 (8). All CT images were independently reviewed by two fellowship-trained cardiothoracic radiologists, and final decisions were reached by consensus.

MuLBSTA Score and CURB 65 Score

The MuLBSTA Score were scaled in all patients. The score points as follows: Multi-lobular infiltrates (5 points), lymphocyte count $\leq 0.8 \times 10^9$/L (4 points), bacterial coinfection (4 points, presented with bacteria positive by laboratory tests or sputum tests and there were consolidation signs on CT feature), acute smoker (3 points, and the patients who had quit-smoking history were scaled as 2 points), hypertension (2 points), and age $\geq$ 60 years (2 points). All patients received a total score calculation for MuLBSTA score. A score of 12 points was used as the cutoff value for mortality risk stratification [MuLBSTA 0-11 (low-risk" mortality) and MuLBSTA 12-22 (high-risk mortality)] (9).

Confusion, Urea, Respiratory rate, Blood pressure, Age 65 (CURB65) score were also scaled. The CURB65 is recommended for assessing the severity of pneumonia in hospital settings and the score system refer to previous studies (10, 11).

Clinical Outcomes

Complications such as electrolyte disturbance, acute myocardial injury (AMI), acute kidney injury (AKI), acute respiratory distress syndrome (ARDS), and shock were recorded. The time from onset to admission and from admission to discharge were also recorded. Clinical outcomes included Death, ICU care, and recovery/discharge.

TABLE 1 | Baseline, clinical treatment, and outcome of all patients.

Baseline	Total ($N = 117$)	Treatment	Total ($N = 117$)
Age (years)		**Severity type**	
<40	37 (31.6%)	Common type	81 (69.2%)
40–59	45 (38.5%)	Severe type	16 (13.7%)
≥60	35 (29.9%)	Critically severe type	20(17.1%)
Male	55 (47.0%)	**Support Treatment**	
Hypertension	19 (16.2%)	High flow oxygen	19 (16.2%)
Diabetes	18 (15.4%)	Non-invasive Invasive	7 (6.0%) 6 (5.1%)
CVD	8 (6.8%)	CRRT	9 (7.7%)
CKD	5 (4.3%)	ECMO	1 (0.8%)
Obesity	16(13.8%)		
Clinical outcome			
ICU care	21 (17.9%)	**Medicine treatment**	
Discharged	96 (82.1%)	Antiviral	105 (89.7%)
Death	5 (4.3%)	Antibiotic	26 (22.2%)
Interval time		Thymalfasin	13 (11.1%)
Onset to admission (days)	5 (3–7)	Chinese medicinal	81 (69.2%)
Onset to discharge (days)	16 (14–23)	Interferon	104 (88.9%)
Admission to discharge (days)	12 (10–15)	Convalescent plasma	8 (6.8%)

Date are n (%), mean ± SD, or median (IQR). CVD, cardiovascular disease; ICU, intensive care unit; CRRT, continuous renal replacement therapy; ECMO, extracorporeal membrane oxygenation.

TABLE 2 | The clinical characteristics and CT feature in different type patients.

	Total *N* = 117	Common type *N* = 81	Severe type *N* = 36
BMI	23.5 ± 3.8	23.4 ± 3.9	23.7 ± 3.9
CURB65 score	0.6 ± 1.0	0.2 ± 0.5	1.4 ± 1.2*
Prone positioning	12 (33.3%)	0 (0%)	12 (33.3%)
Laboratory			
Lymphocyte ($10^{9/}$)	1.4 ± 1.2	1.7 ± 1.3	0.9 ± 0.4*
Decreased	34 (29.1%)	13 (16.0%)	21 (58.3%)
Lymphocyte rate (%)	22.9 ± 11.3	27.8 ± 9.9	15.5 ± 7.9*
Decreased	45 (38.5%)	17 (19.1%)	28 (77.8%)*
CRP (mg/L)	20.1 ± 30.9	10.5 ± 21.3	38.7 ± 36.4*
Increased	67 (57.3%)	34 (42.0%)	33 (91.7%)*
CT imaging			
Interval time from symptoms onset to CT (days)	11 (6–18)	10 (6–17)	13 (9–20)
≥3 Lung lobes affected	82 (70.1%)	48 (59.3%)	34 (94.4%)*
> 2 Mixture signs	88 (75.2%)	53 (59.6%)	35 (97.2%)*
Total lung severity	4.7 ± 3.6	3.3 ± 2.4	7.8 ± 3.9*

**P < 0.05 vs. common type. Date are n (%), mean ± SD, or median (IQR). CT, computerized tomography; BMI, body mass index; CURB65, confusion, urea, respiratory rate, blood pressure, age 65; CRP, Creative protein.*

TABLE 3 | The MuLBSTA score and complications in different type patients.

	Total *N* = 117	Common type *N* = 81	Severe type *N* = 36
MuLBSTA score	8 ± 5	6 ± 4	11 ± 5*
≥12	18 (15.4%)	0 (0)	18 (50.0%)*
Age ≥60 years	35 (29.9%)	15 (18.5%)	20 (55.6%)*
Hypertension	19 (16.2%)	5 (6.2%)	14 (38.9%)*
Smoker	11 (9.4%)	4 (4.9%)	7 (19.4%)
Lymphocyte $<0.8*10^9$/L	33 (28.2%)	13 (16.0%)	20 (55.6%)
Multi-lobular infiltrates	66 (56.4%)	36 (44.4%)	30 (83.3%)
Bacterial coinfection	28 (23.9%)	9 (11.1%)	19 (52.8%)*
Complications			
Electrolyte disturbance	31 (26.5%)	12 (14.8%)	19 (52.8%)*
AMI	12 (10.3%)	2 (2.5%)	11 (30.6%)*
Respiratory failure	17 (14.5%)	0 (0%)	17 (47.2%)*
AKI	3 (2.6%)	1 (1.2%)	2 (5.5%)
ARDS	5 (4.3%)	0 (0%)	5 (13.9%)
Shock	4 (3.4%)	0 (0%)	4 (11.1%)

**P < 0.05 vs. common type. AMI, acute myocardial injury; AKI, acute kidney injury; ARDS, acute respiratory distress syndrome.*

Statistical Analysis

All tests were two-sided, and $P < 0.05$ was considered statistically significant. Categorical variables were described as frequency rates or percentages, and continuous variables were presented as mean (SD) or median (IQR). The mean values for continuous variables were compared using the independent *t*-tests when the data were normally distributed; otherwise, the Mann–Whitney test was used. For laboratory results, we also assessed whether the measurements were outside the normal range. The ROC curve was used to examine the efficacy of the MuLBSTA score for death or ICU. The Kaplan–Meier (K–M) survival analysis was performed to estimate the survival probabilities for COVID-19 infection by the log-rank test. All statistical analyses were performed using 22.2 SPSS software (Statistical Package for the Social Sciences).

RESULTS

Presenting Characteristics

The study population included 117 hospitalized patients with confirmed cases of COVID-19. The youngest patient was 3 months old. Furthermore, 2 patients were admitted with mild symptoms and classified as common type, but then were classified as critically severe type after admission due to the symptoms rapidly aggravated. Moreover, there were 35 (29.9%) patients were older than 60 years. A total of 55 (47.0%) patients were men. The underlying diseases showed in **Table 1**. For the clinical severity type, 81 (69.2%) patients were common type, and 16 (13.7%) and 20 (17.1%) were severe and critically severe types, respectively. Among all patients, most of them (96, 82.1%) were discharged, 21 (17.9%) were admitted to the ICU, and 5 (4.3%) died.

Of all 117 patients, the symptomatic treatment and invasive treatment were shown in **Table 1**. Among of nine patients with continuous renal replacement therapy, three were acute kidney injury, six were electrolyte disturbance or hypercytokinemia (2 patients had concurrent both of two conditions). And five patients were chronic kidney injury. One received extracorporeal membrane oxygenation due to the condition continues to deteriorate. Otherwise, the medicine treatment were also showed in **Table 1**, there were 8 (6.8%) patients repeatedly tested PCR positive, stayed in the hospital for more than 30 days, and received convalescent plasma (from cared patients).

The Clinical Characteristics and CT Feature in Different Patients

In all patients, there was no statistical difference in body mass index (BMI) between common type and severe type patients. Among all severe type patients, the mean point of CURB65 score was 1.4 ± 1.5. And 12 (33.3%) of the patients underwent the prone position management.

The lymphocyte count and rate in severe type patient were significantly lower than those in common type patients ($P < 0.05$). Among the severe type patients, 21 (58.3%) and 28 (7.8%) presented with decreased lymphocyte count and rate, respectively, of which the percentages were higher than those in common type patients. Furthermore, the increased CPR level was higher in severe type patients than in common type patients (33 [91.7%] vs. 34 [42.0%]).

For the assessment of CT features, in all of 36 severe type patients, 34 (94.4%) and 35 (97.2%) showed more than 3 lung lobes affected and more than 2 mixture signs, respectively. The lung lobes involvement was shown in **Table 2**. Severe type patients had significantly higher lung severity scores than common type patients (7.8 ± 3.9 vs. 3.3 ± 2.4, $P < 0.05$).

The Scores and Clinical Complications

A total of 18 patients had a MuLBSTA score >12 points and were all of severe type (**Table 3**). In the severe type patients, 20 (55.6%) were older than 60 years, 14 (38.9%) had hypertension, 7 (19.4%) were smokers, 20 (55.6%) had a lymphocyte count of $<0.8*10^9$/L, 30 (83.3%) had multi-lobular infiltrates, and 19 (52.8%) had bacterial coinfection. The frequency of all the terms in severe type patients was higher than that in the common type patients.

For CURB65 score, the mean point in severe type patients were significantly higher than common type patients ($P < 0.05$). Among of 36 severe type patients, there were 16 patients and 10 patients were more than 2 points and 3 points, respectively. In ICU care and death patients, there were 4 (19.0%) patients and 2 (40%) patients had CURB65 score more than 3 points. The proportion of MuLBSTA score more than 12 points was much higher compared with the proportion of CURB65 score more than 3 points in ICU care and deaths ($P < 0.05$) (**Figure 1**).

During admission, the complications of severe type patients were as follows: 19 (52.8%) patients had with electrolyte disturbance; 11 (30.6%) with AMI; 17 (47.2%) with respiratory failure; 2 (5.5%) with AKI; and 5 (13.9%) with ARDS. Moreover, 4 (11.1%) patients experienced shock and were all of severe type. The frequency of electrolyte disturbance, AMI and respiratory failure in severe type patients were higher than common type patients. The hypokalemia and respiratory failure type I were most common (**Table 3**).

Efficacy and Prognosis Value of the MuLBSTA Scale for Death or ICU Care

Of all 21 patients who required ICU care, 13 (61.9%) and 16 (71.9%) had a MuLBSTA score >12 points. The median point of the MuLBSTA score was 13 (IQR, 9, 15). All (100%) patients who died had a MuLBSTA score ≥ 12 points, and the median point was 17 (IQR, 14, 17).

The diagnosis of the MuLBSTA score for death or ICU treatment is shown in **Figure 2**. The area under the curve (AUC) of death diagnosis was 0.956, the cutoff value was 12 (specificity, 89.5%; sensitivity, 100%). The AUC of ICU diagnosis was 0.875, and the cutoff value was 11 (specificity, 91.7%; sensitivity, 71.4%).

The subgroup analysis of the association between the MuLBSTA score and death or ICU care patients were showed in **Figure 3.** Patients with a MuLBSTA score ≥ 12 had a higher ICU care (log-rank, $P = 0.001$) and higher death (log-rank, $P = 0.000$) risks. The decreasing number of patients at high risk group and the total number of deaths accumulated over time and ICU admissions in the cohort are shown in **Figure 3**.

DISCUSSION

In this cohort study, we reported the clinical characteristics and available risk stratification scores associated with the clinical outcomes in patients with COVID-19 pneumonia who died or

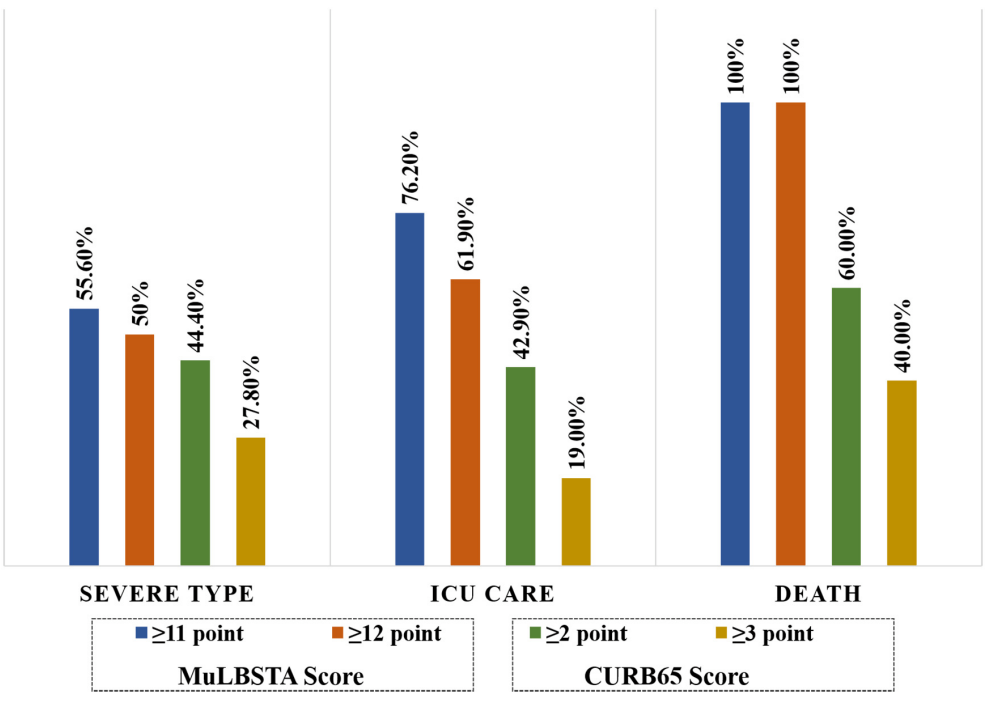


FIGURE 1 | The proportion of different MuLBSTA and CURB65 score points in severe type patients.

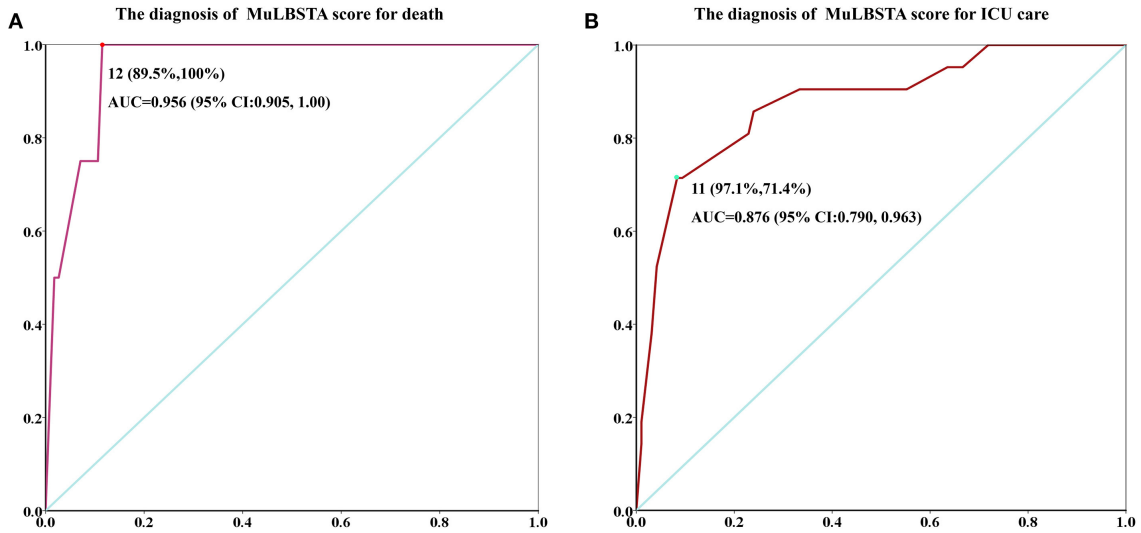


FIGURE 2 | ROC curve of the MuLBSTA score. **(A)** The AUC of death. **(B)** The AUC of ICU.

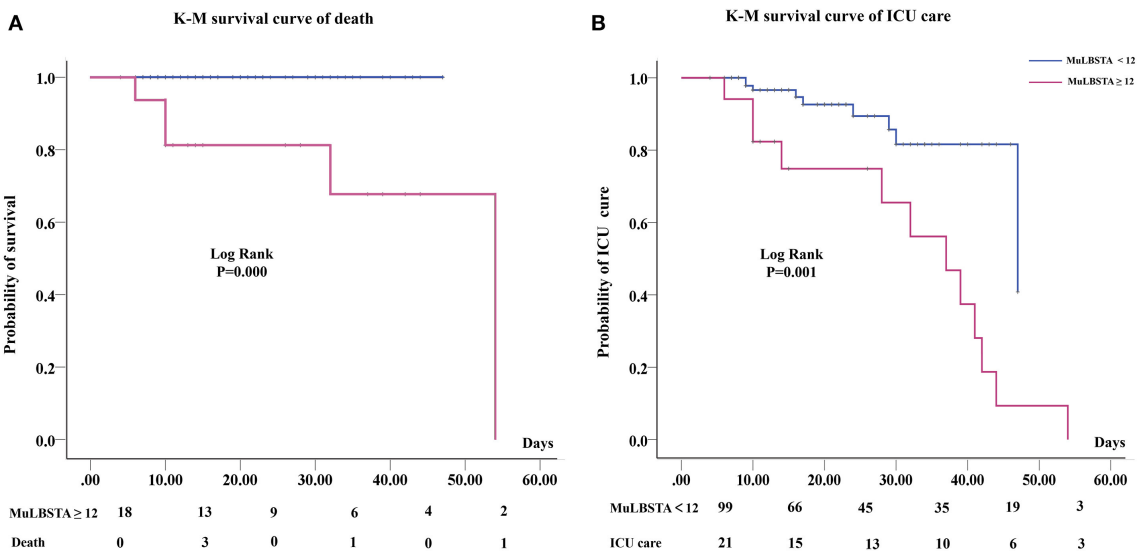


FIGURE 3 | K–M curve of different MuLBSTA scores patients and count of patients in each group at risk at each interval. **(A)** Death risk of MuLBSTA score of ≥12; **(B)** The ICU care needs of MuLBSTA score of ≥12.

required ICU care after admission. Patients with a MuLBSTA score ≥12 points were more likely to die or require ICU care. Particularly, severe type patients were more likely to be older, associated with more underlying diseases, severe immune response and lung involvement. These findings suggest that for patients with COVID-19, the MuLBSTA score at first-time hospital admissions may be necessary for risk stratification in patients who have poor prognosis.

As a new type of highly contagious disease in human, this is the first coronavirus to cause a human pandemic (12). The pathophysiology and risk factors of unusually high mortality for COVID-19 have not yet been completely understood. In this study, we validated an effective clinical risk stratification scoring scale-MuLBSTA score for patients infected with SARS-COV-2. This scale is based on the mortality outcomes of 528 patients infected with respiratory viruses according to Guo et al. (9). However, there is no sufficient evidence to verify the efficacy of assessment of poor prognosis in COVID-19 patients (13). The scale is used as an early warning model in predicting mortality in viral pneumonia (9). This scale synthesizes multiple risk factors of the patient, and finally obtains a total score according to the proportions of different risk factor, which is equivalent to the score of the patient's basic condition.

Old age and underlying diseases are now well-known as risk factors in COVID-19 patients, and it has been reported that the SARS-COV-2 infection was more like to occur in older men with comorbidities (13–15). Wu et al. thought that older age was associated with a greater risk of developing ARDS and death, and it may be owing to less rigorous immune response (16). In their cohort, 29.9% of patients were older than 60 years, and 16.2 and 15.4% had associated with hypertension and diabetes. Hypertension and CVD had higher prevalence in the severe cases than in the mild ones. Moreover, no study have demonstrated that a single underlying illness is a risk factor for death or treatment in the ICU at present. Old age or age and underlying disease alone may not be sufficient to determine the risk. In earlier reports increased age in the male population has been associated with higher mortality (17). Smokers are vulnerable to respiratory viruses, and smoking could upregulate angiotensin-converting enzyme 2 receptor levels (17). The prevalence of high smoking level in males may partly explain the higher susceptibility and mortality of male patients.

In addition, virus-induced direct cytopathic effects and viral evasion of host immune responses are believed to play major roles in the severity of coronavirus infection (18, 19). The dysregulation of immune response may result in an excessive inflammation, leading to adverse outcomes (20, 21). Lymphocytopenia was present in 83.2% patients with COVID-19 at admission (22), and severe cases tend to have lower lymphocyte counts (5). In this study, we had similar findings that lymphocyte counts significantly decreased in severe type patients, and more than half of patients had decreased lymphocyte count. Coronaviruses commonly attack the respiratory system and SARS-CoV-2 has been shown to cause lung damage (22, 23). As a significant auxiliary modality, chest CT is a key component of the diagnosis of virus-infected patients (24). It allows the sensitive assessment of lung lesions as well as the degree and location of lung involvement. In previous studies, ground glass and consolidation opacities have been shown to be the most common imaging signs in patients with COVID-19 (8, 23, 25). Although weakened immunity and lung damage are problems in the majority of patients, the effect on death or ICU remains unclear.

The MuLBSTA score is a good diagnostic marker for poor prognosis. In the present study, a score of 12 points indicates the specificity and sensitivity of death were 89.5 and 100%, and 11 points present the specificity and sensitivity of ICU care were 91.7 and 71.4%, respectively. These results strongly suggest that the scale has good efficacy to assess the clinical risk of death and ICU care in patients infected with SARS-COV-2. The survival analysis showed that the higher is the MuLBSTA score, the higher is the death risk. In our results, 50% of severe type patients had a score of $\geq$12 points, but no common type patients had more than this score. The results implied that severe type patients are more likely to die, and it may be owing to severe immune response and lung involvement.

In clinical practices, there is no effective treatment available for the infected patients, but screening high-risk patients at first admission and appropriate clinical management may be helpful in reducing the incidence of severe complications, such as ARDS or sepsis as well as mortality. Although there are some clinical scales about the severity and risk stratification of pneumonia, such as CURB65 or SOFA score, but in our study, the screening proportion of high-risk patients with MuLBSTA score was higher than that of CURB65 score. Meanwhile, age, hypertension, and smoking status as part of the MuLBSTA score were readily available in the clinical setting, whereas the lymphocyte count and lobe status were assessed by routine blood examination and X-ray or CT scan. Therefore, the score may be a rapid and effective risk stratification strategy.

This study has several limitations. First, the lack of effective antiviral drugs, and all patients underwent different treatment regimens, which may affect the prognosis of patients. Secondly, there may be other risk factors that also affect the prognosis of patients. We verified the validity of the MuLBSTA scale, but the predictive value of a single factor was not analyzed. Finally, because this is the retrospective study, we could only evaluate the short-term prognosis. The long-term prognosis would be analyzed in further studies.

To the best of our knowledge, this is the first retrospective cohort study that focusing on the MuLBSTA score risk stratification of patients with COVID-19 who have experienced a definite outcome. We found that a MuLBSTA score of $\geq$12 points at admission was a high risk factor for death or ICU care in adult patients with COVID-19. The risk stratification provides the evidence for novel coronavirus clinical interventions in efforts to improve outcomes.

CONCLUSIONS

A higher MuLBSTA score at admission had higher death or ICU risk in patients with COVID-19. The MuLBSTA scale is valuable for the risk stratification of COVID-19 patients, especially regarding death or ICU care.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

The study was approved by the hospital ethics committee. Written informed consent was waived owing to the rapid emergence of this infectious disease.

AUTHOR CONTRIBUTIONS

MY and YG designed the study and takes responsibility for the integrity and accuracy of the data analysis. RX, KH, and KZ contributions to the acquisition, analysis, interpretation of data for the work, and writing of the manuscript. RX, KH, KZ, HX, NZ, HF, LX, RS, LW, HL, and ZY had roles in patient recruitment, data collection, and clinical management. All authors contributed to data acquisition, data analysis, and all reviewed and approved the final version of the manuscript.

FUNDING

This work was supported by 2020 Novel coronavirus pneumonia prevention and control technology project of Chengdu (No. 2020-YF05- 00007-SN); Clinical research finding of Chinese society of cardiovascular disease (CSC) of 2019 (HFCSC2019B01); the National Natural Science Foundation of China (Nos. 81771887, 81771897, 81971586, and 81901712); the Program for Young Scholars and Innovative Research Team in Sichuan Province (No. 2017TD0005) of China; and 1·3·5 project for disciplines of excellence, West China Hospital, Sichuan University (No. ZYGD18013).

ACKNOWLEDGMENTS

We thank all our colleagues who helped us during the current study. We are also grateful to the many front-line medical staff for their dedication in the face of this outbreak, despite the potential threat to their own lives and the lives of their families.

REFERENCES

1. Wu ZY, McGooga JM. Characteristics of and important lessons from the Coronavirus Disease 2019 (COVID-19) outbreak in China summary of a report of 72314 cases from the Chinese center for disease control and prevention. *JAMA*. (2020) 323:1239–42. doi: 10.1001/jama.2020.2648
2. WHO. *WHO Director-General's Opening Remarks at the Media Briefing on COVID-19 - 3 April 2020*. (2020). Available online at: https://www.who.int/dg/speeches/detail/who-director-general-s-opening-remarks-at-the-media-briefing-on-covid-19--3-april-2020 (accessed April 3, 2020).
3. Cao Y, Liu X, Xiong L, Cai K. Imaging and clinical features of patients with 2019 novel coronavirus SARS-CoV-2: a systematic review and meta-analysis. *J Med Virol*. (2020). doi: 10.1002/jmv.25822. [Epub ahead of print].
4. Mahase E. COVID-19: death rate is 0.66% and increases with age, study estimates. *BMJ*. (2020) 369:m1327. doi: 10.1136/bmj.m1327
5. Qin CN, Zhou LQ, Hu ZW, Shoqi Z, Sheng Y, Yu T, et al. Dysregulation of immune response in patients with COVID-19 in Wuhan, China. *Clin Infect Dis*. (2020) 71:762–8. doi: 10.2139/ssrn.3541136
6. Graziano O, Giovanni R, Silvio B. Case-fatality rate and characteristics of patients dying in relation to COVID-19 in Italy. *JAMA*. (2020) 323:1775–6. doi: 10.1001/jama.2020.4683
7. Liu J, Zheng X, Tong Q, Li W, Wang B, Sutter K, et al. Overlapping and discrete aspects of the pathology and pathogenesis of the emerging human pathogenic coronaviruses SARS-CoV, MERS-CoV, and 2019-nCoV. *J Med Virol*. (2020) 92:491–4. doi: 10.1002/jmv.25709
8. Michael C, Adam B, Xueyan M, Ning Z, Mingqian H, Zeng X, et al. CT Imaging features of 2019 Novel Coronavirus (2019-nCoV). *Radiology*. (2020) 295:202–7. doi: 10.1148/radiol.2020200230
9. Guo L, Wei D, Zhang X, Wu Y, Li Q, Zhou M, et al. Clinical features predicting mortality risk in patients with viral pneumonia: the MuLBSTA score. *Front Microbiol*. (2019) 10:2752. doi: 10.3389/fmicb.2019.02752
10. Lim WS, van der Eerden MM, Laing R, Boersma WG, Karalus N, Town GI, et al. Defining community acquired pneumonia severity on presentation to hospital: an international derivation and validation study. *Thorax*. (2003) 58:377–82. doi: 10.1136/thorax.58.5.377
11. Lim WS, Baudouin SV, George RC, Hill AT, Le Jeune I, Macfarlane JT, et al. BTS guidelines for the management of community acquired pneumonia in adults: update. *Thorax*. (2009) 64(Suppl 3):iii1–55. doi: 10.1136/thx.2009.121434
12. WHO. Available online at: https://www.who.int/dg/speeches/detail/who-director-general-s-opening-remarks-at-the-mission-briefing-on-covid-19-$-$2-april-2020 (2020). (accessed April 2, 2020).
13. Chen N, Zhou M, Dong X, Qu J, Gong F, Han Y, et al. Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study. *Lancet*. (2020) 395:507–13. doi: 10.1016/S0140-6736(20)30211-7
14. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet*. (2020) 395:497–506. doi: 10.1016/S0140-6736(20)30183-5
15. Li JY, You Z, Wang Q, Zhou ZJ, Qui Y, Luo R, et al. The epidemic of 2019-novel-coronavirus (2019-nCoV) pneumonia and insights for emerging infectious diseases in the future. *Microbes Infect*. (2020) 22:80–5. doi: 10.1016/j.micinf.2020.02.002
16. Wu CM, Chen XY, Cai YP, Xia J, Xu S, Huang H, et al. Risk factors associated with acute respiratory distress syndrome and death in patients with coronavirus disease 2019 pneumonia in Wuhan, China. *JAMA Intern Med*. (2020) 2020:e200994. doi: 10.1001/jamainternmed.2020.0994
17. Brake SJ, Barnsley K, Lu W, McAlinden KD, Eapen MS, Sohal SS, et al. Smoking upregulates angiotensin-converting enzyme-2 receptor: a potential adhesion site for novel coronavirus SARS-CoV-2 (COVID-19). *J Clin Med*. (2020) 9:E841. doi: 10.3390/jcm9030841
18. Min CK, Cheon S, Ha NY, Sohn KM, Kim Y, Aigerim A, et al. Comparative and kinetic analysis of viral shedding and immunological responses in MERS patients representing a broad spectrum of disease severity. *Sci Rep*. 6:25359. doi: 10.1038/srep25359
19. Channappanavar R, Perlman S. Pathogenic human coronavirus infections: causes and consequences of cytokine storm and immunopathology. *Semin Immunopathol*. (2017) 39:529–39. doi: 10.1007/s00281-017-0629-x
20. Shaw AC, Goldstein DR, Montgomery RR. Age-dependent dysregulation of innate immunity. *Nat Rev Immunol*. (2013) 13:875–87. doi: 10.1038/nri3547
21. Guan WJ, Ni ZY, Hu Y, Liang W, Ou C, He J, et al. Clinical characteristics of coronavirus disease 2019 in China. *N Engl J Med*. (2020) 382:1708–20. doi: 10.1056/NEJMoa2002032
22. Hyun JK, Soyeoun L, Jooae C, Choi SH, Sung H, Do KH, et al. Radiographic and CT features of viral pneumonia. *RadioGraphics*. (2018) 38:719–39. doi: 10.1148/rg.2018170048
23. Ai T, Yang ZL, Hou HY, Zhan C, Chen C, Lv W, et al. Correlation of Chest CT and RT-PCR testing in coronavirus disease 2019 (COVID-19) in China: A Report of 1014 Cases. *Radiology*. (2020) 296:E32–40. doi: 10.1148/radiol.2020200642
24. Zu ZY, Jiang MD, Xu PP, Chen W, Ni Q, Lu GM, et al. Coronavirus disease 2019 (COVID-19): a perspective from China. *Radiology*. (2020) 296:200490. doi: 10.1148/radiol.2020200490
25. Shi HS, Han XY, Jiang NC, Cao Y, Alwalid O, Gu J, et al. Radiological findings from 81 patients with COVID-19 pneumonia in Wuhan, China: a descriptive Study. *Lancet Infect Dis*. (2020) 20:425–34. doi: 10.1016/S1473-3099(20)30086-4

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Communication

BRIEF RESEARCH REPORT
published: 14 August 2020
doi: 10.3389/fcomm.2020.00057

Themes and Evolution of Misinformation During the Early Phases of the COVID-19 Outbreak in China—An Application of the Crisis and Emergency Risk Communication Model

*Jiahui Lu**

School of New Media and Communication, Tianjin University, Tianjin, China

Social media has enabled misinformation to circulate with ease around the world during the novel coronavirus disease 2019 (COVID-19) pandemic. This study applies the Crisis and Emergency Risk and Communication model (CERC) to understand the themes and evolution of misinformation on the Internet during the early phases of the COVID-19 outbreak in China, when the epidemic developed rapidly with mysteries. Drawing on 470 misinformation rated as false by three leading Chinese fact-checking platforms between 1 January and 3 February 2020, the analysis demonstrated five major misinformation themes surrounding COVID-19: prevention and treatment, crisis situation updates, authority action and policy, disease information, and conspiracy. Further trend analyses found that misinformation emerged only after the nationwide recognition of the crisis, and appeared to evolve relating to crisis stages, government policies, and media reports. This study is the first to apply the CERC model to investigate the primary themes of misinformation and their evolution. It provides a standard typology for crisis-related misinformation and illuminates how misinformation of a particular topic emerges. This study has significant theoretical and practical implications for strategic misinformation management.

Keywords: COVID-19, misinformation, internet, surveillance, crisis communication, fact-checking

OPEN ACCESS

Edited by:
Rukhsana Ahmed,
University at Albany, United States

Reviewed by:
Raihan Jamil,
Zayed University,
United Arab Emirates
Zhenyi Li,
Royal Roads University, Canada

***Correspondence:**
Jiahui Lu
tkdhuihui28@gmail.com

Specialty section:
This article was submitted to Health Communication, a section of the journal Frontiers in Communication

Received: *14 May 2020*
Accepted: *06 July 2020*
Published: *14 August 2020*

Citation:
Lu J (2020) Themes and Evolution of Misinformation During the Early Phases of the COVID-19 Outbreak in China—An Application of the Crisis and Emergency Risk Communication Model. Front. Commun. 5:57. doi: 10.3389/fcomm.2020.00057

INTRODUCTION

The Internet, especially social media, has caused considerable concerns on their roles in promoting misinformation during health crises like disease outbreaks (Tandoc et al., 2018; Waszak et al., 2018). Misinformation can be broadly defined as "information presented as truthful initially but that turns out to be false later on" (Lewandowsky et al., 2013). It may inhibit effective outbreak communication by amplifying public fear and misleading the public to develop practices that might harm their health (Poland and Spier, 2010; Swire and Ecker, 2018). Particularly, in the current coronavirus disease 2019 (COVID-19) pandemic, the United Nations has warned a

"misinfo-demic" that is spreading harmful health advice on the Internet[1].

A rapidly growing body of literature has investigated the misinformation surrounding disease outbreaks, such as the current COVID-19 pandemic. Research has found that a quarter of social media information (e.g., Twitter, YouTube) contained medical misinformation and unverified content pertaining to the COVID-19 pandemic (Kouzy et al., 2020; Li et al., 2020). Over two-thirds of the top 110 popular websites from the Google Search engine were found to have a low quality of COVID-19 information (Cuan-Baltazar et al., 2020).

Different typologies of misinformation were also identified in previous studies (Bastani and Bahrami, 2020; Brennen et al., 2020). Brennen et al. (2020) found 9 topics of COVID-19 misinformation, with falsehoods about "public authority action," "community spread," "general medical advice," and "prominent actors" as the most prevalent topics. Bastani and Bahrami (2020) identified 5 main categories of misinformation, including "disease statistics," "treatments, vaccines and medicines," "prevention and protection methods," "dietary recommendations," and "disease transmission ways." Similarly, studies surrounding the Ebola and Zika outbreak found misinformation about disease health impacts, vaccinations, and disease transmission mechanisms (Oyeyemi et al., 2014; Sommariva et al., 2018; Vijaykumar et al., 2018).

However, two essential research gaps can be identified. First, none of the studies utilized a theoretical model to guide the development of their misinformation typologies. Particularly, it is essential to develop a topology of crisis-related misinformation based on a crisis communication model. A theoretical typology of crisis-related misinformation can facilitate comparisons and integration of previous findings by providing a standard framework. This can also inform better health communication and in turn improve crisis management.

Second, how misinformation emerges and evolves during disease outbreaks remain unclear, preventing strategic crisis management on combating misinformation. Theories indicate that misinformation like rumors is induced during health crises because of the public's unsatisfied information needs (Rosnow, 1991; DiFonzo and Bordia, 2007). Rumors can often play crucial roles in reducing public feelings of anxiety and uncertainty that are triggered by the unknown and threatening circumstances (Rosnow, 1991; DiFonzo and Bordia, 2007). Hence, as the situations develop swiftly during disease outbreaks like COVID-19, it is likely that different types of misinformation emerge and evolve in different time frames corresponding to the information needs of the public. Nevertheless, none of the existing research has explored the temporal patterns of misinformation.

An Application of the Crisis and Emergency Risk Communication Model

This research aims to address the above research gaps by adopting the Crisis and Emergency Risk Communication Model (CERC) to examine the typology and evolution of misinformation during the early phases of a disease outbreak. The CERC is a communication model that guides authorities' communication strategies at different stages of the risk and crisis lifecycle, "from risk, to eruption, to clean-up and recovery, and on into evaluation (p. 51)" (Reynolds and Seeger, 2005). The latter three stages mark the containment and the end of the crisis, when misinformation may not be prevalent. Therefore, this study focuses only on the first two stages of a health crisis. As the model is developed based on the audiences' information needs (Reynolds and Seeger, 2005), it provides the temporal patterns of those needs that can be used to theorize the typology and evolution of crisis-related misinformation.

The pre-crisis stage is the first stage of the CERC model when the crisis is yet to occur. The authorities will need to heighten the public awareness of the potential crisis by warning the public, providing risk information, and educating self-preventions. However, in the context of crisis-related misinformation, as the CERC model suggests that the public is generally not aware of the crisis at this stage (Reynolds and Seeger, 2005), it can be expected that there will be no misinformation during the pre-crisis stage.

The second stage of CERC is the initial stage when the crisis occurs and when the public is first aware of the crisis. The model demonstrates uncertainty reduction, self-efficacy, and reassurance as the three main communication strategies at this stage (Reynolds and Seeger, 2005). In the context of an infectious disease outbreak (Lazard et al., 2015; Lwin et al., 2018), reducing uncertainty is to provide information on case reports and crisis-related events. Messages on self-efficacy are to communicate personal preventions and treatments. Reassurance is information about government interventions. That is, the initial stage is characterized by three urgent information needs: crisis situation updates, self-preventions, and reassurance from authority-initiated actions. Accordingly, misinformation about crisis situations, self-preventions, and authority actions will be prominent at this stage. Specifically, the public requires more accurate understandings of the disease as the crisis continues to develop (Centers for Disease Control and Prevention, 2018; Lwin et al., 2018). Hence, misinformation about disease natures is expected to surge at the later period of the initial stage.

Though the CERC model suggests that misinformation will develop surrounding the four themes during the initial stage of an outbreak, it does not provide detailed predictions on how these four themes of misinformation emerge and evolve within the stage. It is important to understand the rapid evolution of misinformation because disease outbreaks often develop swiftly with many mysteries within the initial stage. Also, breaking news and government responses will be put forth rapidly within this period. It is crucial to explore if government actions and media reports will affect the emergence of misinformation.

This study takes the COVID-19 outbreak in China as a valuable opportunity for applying the CERC to understand the typology and evolution of crisis-related misinformation. Particularly, this research aims to analyse the themes and temporal patterns of COVID-19 misinformation by utilizing data from fact-checking platforms.

[1]United Nations. Hatred going viral in 'dangerous epidemic of misinformation' during COVID-19 pandemic. Available online at: https://news.un.org/en/story/2020/04/1061682 (accessed June 26, 2020).

The COVID-19 and Fact-Checking Platforms in China

On 31 December 2019, the first cases of COVID-19 were reported in Wuhan, China (Zhu et al., 2020). The disease has been put under national surveillance since 11 January (Tu et al., 2020). A week later, Guangdong, Shanghai, and Beijing reported their first imported cases from Wuhan. As of 23 January, all but two provinces of China reported confirmed cases[2]. The following days witnessed the quarantine of Wuhan and other cities in the Hubei province[3]. The disease continued to spread and has seen a broader outreach in China since 27 January, but the number of daily new cases began to drop after 3 February[4].

The COVID-19 outbreak has triggered massive amounts of misinformation on social media within China. Fake reports on new cases and unverified information about prevention from the disease (e.g., Taking vitamin C can prevent the disease) have been circulated with ease. Fact-checking platforms, which aim to debunk fake news and online falsehoods, provide novel data sources for misinformation surveillance during the outbreak. This study analyses data from three such platforms, including Jiaozhen, Ding Xiang Yuan, and Toutiao.

Jiaozhen is the leading Chinese fact-checking platform that aims to fight against health-related falsehoods[5]. It is jointly run by the Health Communication Working Committee of China Medical Doctor Association and Tencent, the company that hosts the most active social media application, WeChat. This platform is providing real-time information services during the COVID-19 outbreak in China, by curating and reviewing hot topics in public health from news and social media with the help of artificial algorithms. Ding Xiang Yuan[6], which is the leading social networking site for health professionals in China, has developed a fact-checking platform specifically for the COVID-19 outbreak. The platform is specializing in debunking medical-related misinformation surrounding the disease. Finally, Toutiao is the Chinese leading online news media. It provides services to counter fake news during the crisis by gathering falsehoods from news media[7].

Hypotheses and Research Questions

H1: Misinformation will emerge surrounding crisis situation updates, prevention and treatment, authority action and polity, and disease information during the initial stage of the COVID-19 outbreak in China.

RQ1: How did different themes of misinformation emerge during the first two stages of the COVID-19 outbreak suggested by the CERC model?

METHODS

Data Extraction

All fact-checking articles published between 1 January and 3 February 2020 were extracted from the three platforms, namely Jiaozhen, Ding Xiang Yuan, and Toutiao. The investigation period marked the first two stages of the outbreak. The pre-outbreak stage was from 1 January to 20 January; the initial stage was from 21 January till the end of the investigation. The two stages were categorized as above because the China government recognized the COVID-19 crisis by confirming human-to-human transmission of COVID-19 and starting daily national reports on the outbreak on 20 January, and the disease began to be contained after 3 February[4]. The extraction yielded 524 articles, of which 225 on Jiaozhen, 69 on Ding Xiang Yuan, and 230 on Toutiao.

Data Coding Procedures

Data coding of the articles involved several procedures. First, the author and a student assistant independently scanned all articles with their titles and full texts and identified 470 articles that are related to the COVID-19 outbreak. The interrater agreement of the scanning was 100%. Of those relevant articles, 434 rated their fact-checked stories as false. These include 155 articles on Jiaozhen, 64 on Ding Xiang Yuan, and 215 on Toutiao.

Second, the 434 articles were then thematically analyzed with two steps. In step 1, a codebook was developed by the author. The CERC was applied to develop the typology of COVID-19 related misinformation. As discussed, four major themes were derived from the CERC (Reynolds and Seeger, 2005; Lwin et al., 2018): (1) prevention and treatment: misinformation on measures or medication for disease prevention or treatment; (2) crisis situation updates: misinformation on updates or events related to new or existing cases, or other crisis-related situations; (3) authority action and policy: misinformation on government policies taken against the disease or other public policies; and (4) disease information: misinformation pertaining to disease spreading mechanisms, diagnosis, and other disease-related information.

To ensure all themes of misinformation would be captured, an open coding procedure was also employed (Bernard et al., 2016). The fifth theme was derived from the open coding procedure: (5) conspiracy: false statements or accusations that the virus is human-made or that some countries utilized the virus as a bioengineered weapon. The author and the student assistant then read the posts independently and identified finer topics under each of the five themes. The team met to discuss disagreements and agreed on the final codebook.

In step 2, the author and another student assistant independently went through the title and full text of each article and categorized them into themes and topics guided by the

[2]National Health Commission of the People's Republic of China. Situation reports. Available online at: http://www.nhc.gov.cn/xcs/yqtb/list_gzbd.shtml (accessed June 26, 2020).

[3]General Office of Hubei Provincial People's Government. Wu Han Shi Xin Xing Guan Zhuang Bing Du Gan Ran De Fei Yan Yi Qing Fang Kong Zhi Hui Bu Tong Gao (Di Yi Hao) Statement on the novel coronavirus infection pneumonia outbreak preparedness in Wuhan (No. 1). Available online at: http://www.gov.cn/xinwen/2020-01/23/content_5471751.htm (accessed June 26, 2020).

[4]COVID-19 Dashboard by the Center for Systems Science and Engineering (CSSE) at Johns Hopkins University. Available online at: https://coronavirus.jhu.edu/map.html (accessed June 26, 2020).

[5]Jiaozhen. Available onlin at: https://vp.fact.qq.com/home (accessed June 26, 2020).

[6]Ding Xiang Yuan. Available onlin at: http://ncov.dxy.cn/ncovh5/view/pneumonia_rumors?from=dxyandsource=andlink=andshare= (accessed June 26, 2020).

[7]Toutiao. Available onlin at: https://www.toutiao.com/c/user/62596297771/#mid=1585938857001998 (accessed June 26, 2020).

TABLE 1 | Themes and examples of misinformation.

Themes	Example	Jiaozhen	Ding Xiang Yuan	Toutiao	Sum	%
Prevention and treatment		**66**	**38**	**33**	**137**	**31.6%**
Folk medicine	Taking Vitamin C can prevent the novel coronavirus disease.	30	14	13	57	13.1%
Folk measures	Taking a hot bath can prevent the novel coronavirus disease.	13	11	7	31	7.1%
Clinical treatment	Good news! The vaccine for the novel coronavirus disease is now ready.	13	5	9	27	6.2%
Mask	People should wear a surgical mask with the colored surface facing out when they show respiratory symptoms and with that surface facing internally when they are well.	10	8	4	22	5.1%
Crisis situation updates		**30**	**2**	**89**	**121**	**27.9%**
Case updates	Dr. Zhong Nanshan confirmed with the coronavirus disease.	11	1	43	55	12.7%
Local events	A Hubei news anchor put on a surgical mask when she was broadcasting the news on-screen.	12	1	31	44	10.1%
Local corruption	The Wuhan Red Cross was found to sell vegetables that were donated by the general public.	3	0	7	10	2.3%
Foreign events	116 visitors from Wuhan were refused entry into Singapore.	4	0	8	12	2.8%
Authority action and policy		**33**	**2**	**67**	**102**	**23.5%**
Isolation controls	The Guangzhou government plans to put the city under quarantine.	17	1	50	68	15.7%
Medical measures	The Wuhan government plans to disinfect the city with a large-scale spray.	5	1	6	12	2.8%
Medical supplies management	The state council bans sale of face masks on all eCommerce platforms.	4	0	4	8	1.8%
Contact tracing	From 3 February, all visitors should make registrations with their real name before entering into hospitals in Shanghai.	3	0	3	6	1.4%
Media management	Wuhan plans to block the Internet connection to stop medical staff from sharing disease-related information to the public.	2	0	3	5	1.2%
Reassurance	National public health emergency response: House rental will be fully free for February and halved for March and April.	2	0	0	2	0.5%
General	The city of Hezhou announces the highest level of the public health emergency for the coronavirus.	0	0	1	1	0.2%
Disease information		**23**	**20**	**21**	**64**	**14.7%**
Spreading dynamics	The coronavirus can be transmitted through eye contact.	11	13	6	30	6.9%
Natural history	The coronavirus has been discovered since 2018.	6	3	8	17	3.9%
Risk factor	Asians are easier to get the coronavirus infection.	4	4	5	13	3.0%
Epidemiology	The biggest outbreak is coming in 2 days. Stay home.	1	0	2	3	0.7%
Diagnosis	If one sees symptoms of runny nose and expectoration, he or she is not infected by the coronavirus.	1	0	0	1	0.2%
Conspiracy	The coronavirus is a human-made, bioengineered disease.	**3**	**2**	**5**	**10**	**2.3%**
N		**155**	**64**	**215**	**434**	

The bold values indicates the major themes of misinformation, whose counts and percentages should be the sum of values of its subtopics.

codebook. The interrater agreement between the two coders was 87.1%. The coding disagreement was solved by discussions.

RESULTS

Themes of Misinformation (H1)

Table 1 demonstrates the five themes and examples of misinformation. Several topics were identified under the major themes. First, the theme of "prevention and treatment" was predominant, accounting for 31.6% of all misinformation. "Folk medicine" surfaced as one of the most prevalent topics, presenting folk beliefs of alternative medicines such that taking vitamin C can prevent people from the disease. The second common theme was misinformation pertaining to "crisis situation updates," representing a share of 27.9%. Examples in this theme included fake reports that claimed someone had confirmed with the infection or died of the virus.

"Authority action and policy" emerged as another type of misinformation that was widely spreading (23.5%). Particularly, fake news of isolation controls claiming city quarantine plans was the most frequent topic, making up 15.7% of all misinformation. The fourth common theme was "disease information" (14.7%). An example of this theme is the rumor that the COVID-19 could be transmitted through eye contact between people. Conspiracy (2.3%), such that the coronavirus was bioengineered in the lab, was also circulated extensively within China (Cohen, 2020).

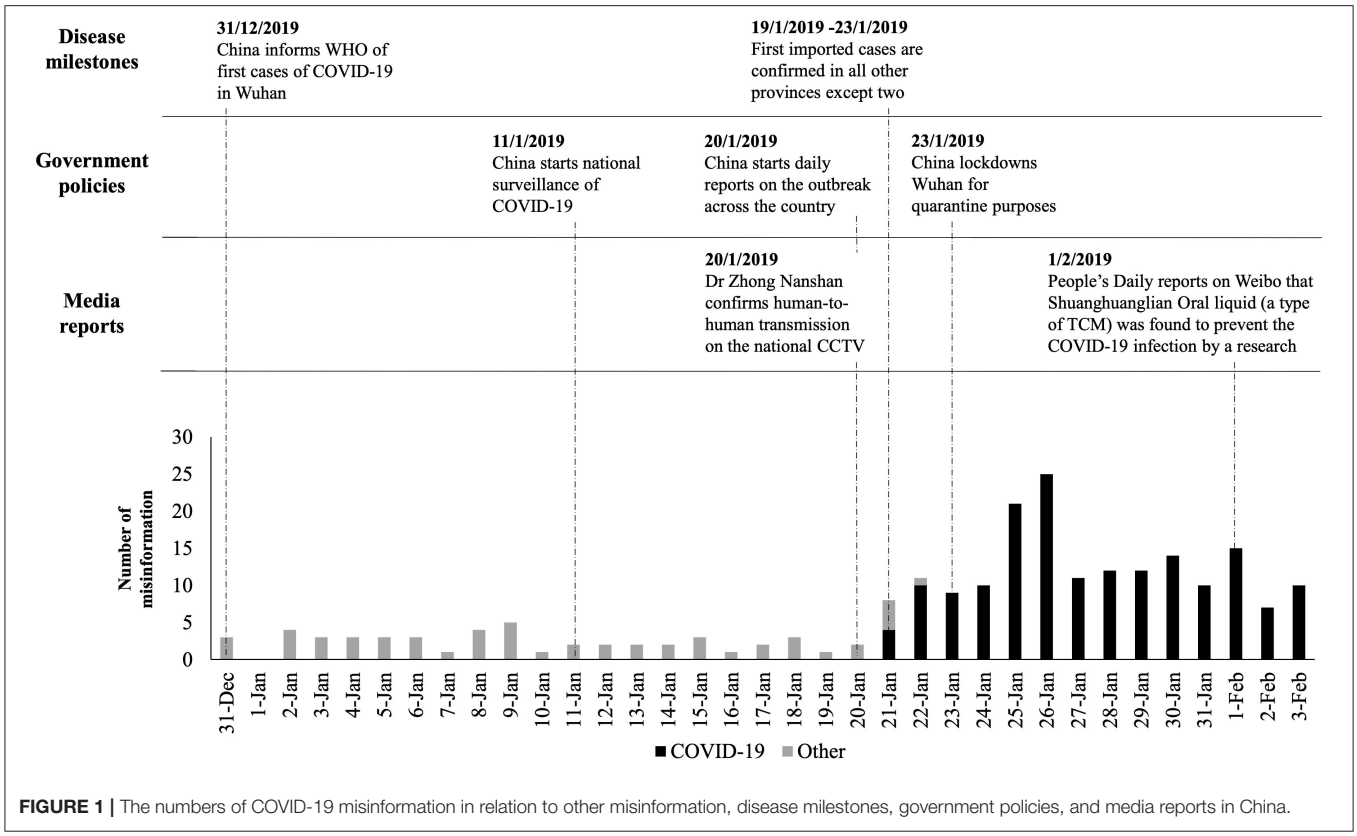


FIGURE 1 | The numbers of COVID-19 misinformation in relation to other misinformation, disease milestones, government policies, and media reports in China.

Evolution of Misinformation (RQ1)

A trend analysis was conducted utilizing Jiaozhen as the only data source. The platform aims to provide timely facts within 24 h after a piece of impactful misinformation emerges online. As such, its data allows investigations of the evolution of misinformation. However, data from the other two platforms are not optimal for trend analysis. Ding Xiang Yuan did not provide a timestamp for its articles, and Toutiao revealed a significant time lag of fact-checking during the investigation period.

Figure 1 shows the numbers of COVID-19 misinformation checked by Jiaozhen between 1 January and 3 February 2020. Fact-checking articles that are not related to the disease were also examined for comparison purposes, presenting the baseline fact-check frequency of the platform. Though the virus infections have been put under national surveillance as early as 11 January, misinformation pertaining to the disease emerged only on 21 January, the day when the crisis was nationally recognized. The number of misinformation peaked at the first 2 days of Chinese New Year (i.e., 25 and 26 January).

Figure 2 demonstrates how different themes of misinformation developed during the initial stage of the outbreak. Particularly, three short meaningful phases were identified within this stage based on disease developments and government actions. The preparation phase (Phase 1; 21–23 January) witnessed the period when the disease was first recognized as a national crisis by the public. Misinformation of prevention and treatment (e.g., folk medicine) and crisis situation updates (e.g., case reports) was predominant at this phase.

The developing phase (Phase 2; 24–27 January) started from the day when the Wuhan city was quarantined. The number of misinformation surrounding authority actions and policies surged at this phase, becoming the most frequent theme. Rumors of isolation measures accounted mostly for the increase.

The escalating phase (Phase 3) has seen an upsurge of confirmed cases across the country from 27 January to 3 February. Mysteries surrounding the disease, especially its spreading dynamics, become salient. Interestingly, on 1 February, People's Daily, the official newspaper of the Central Committee of the Communist Party of China, reported a study that argued the effectiveness of Shuanghuanglian Oral Liquid, one of traditional Chinese medicine that is familiar to the public, in preventing the disease infection. This media report coincided with an escalation of misinformation on folk medicine.

DISCUSSION

This study is the first to apply the CERC model to investigate the primary themes of misinformation and their evolution during the early stages of the COVID-19 outbreak in China. Across three platforms, the study demonstrated five major themes of misinformation. The four predominant themes were derived from the CERC model, including crisis situation updates, prevention and treatment, authority action and polity, and disease information, supporting H1. From a

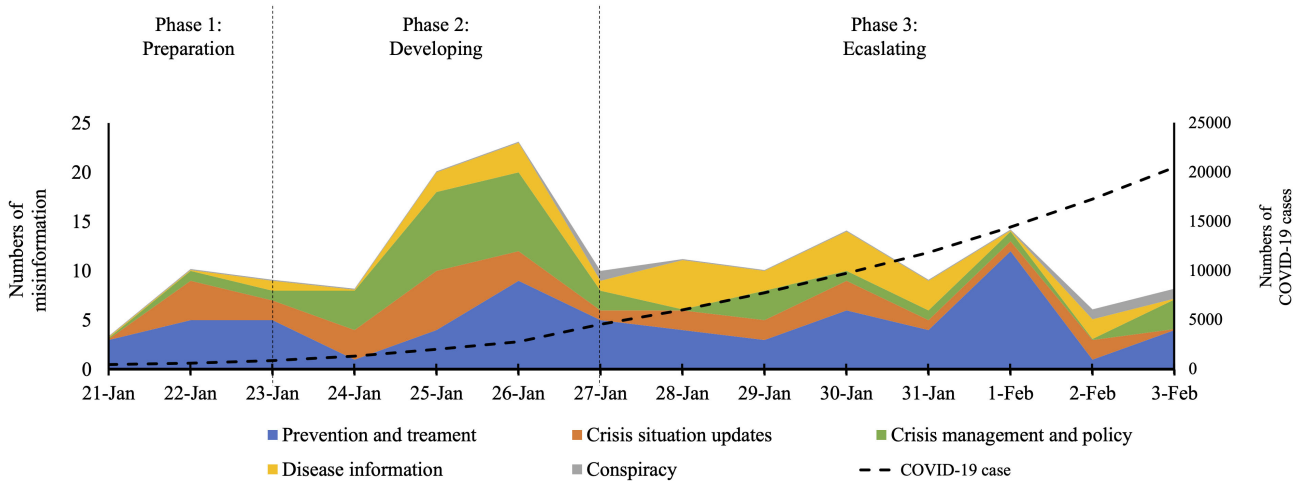


FIGURE 2 | Evolution of misinformation themes in relation to crisis phases and the COVID-19 cases in China.

health communication perspective, this study provides a standard typology for crisis-related misinformation. This is helpful as the framework can guide future systematic reviews to summarize and compare previous findings. From the crisis communication perspective, the findings suggest that combating crisis-related misinformation and communicating crisis information are two sides of a coin. Though this insight seems intuitive, it illuminates the theoretical possibilities for future integration for the two distinct research fields. It also suggests that the containment of crisis-related misinformation should be implemented simultaneously along with crisis communication.

However, the CERC model did not predict the emergence of conspiracies, though conspiracies were commonly found during health crises (Sommariva et al., 2018; Wood, 2018). This is likely because conspiracies do not emerge from a particular information need; instead, it serves to provide an immediate and holistic understanding of the situation: why the crisis happened, who benefits from it, and who should be blamed (Bessi et al., 2015; Wood, 2018). Though conspiracies accounted for only a tiny proportion of misinformation, they can significantly tarnish the reputations of health authorities and prevent effective health and crisis communication (Cohen, 2020). Future studies should investigate how they can be efficiently prevented and addressed during health crises.

Regarding temporal patterns (RQ1), misinformation emerged only after the national recognition of the crisis, supporting the intuitive prediction from the CERC. Importantly, government policies and media reports appear to elicit misinformation under some circumstances at the initial stage of the COVID-19 outbreak in China (21 January to 3 February). The findings clearly showed the concurrence between the city quarantine and the upsurge of fake news about government policies, and between People Daily's reports and the circulation of misinformation about folk medicine. Given the relatively short investigation period in this study, the causality of their associations cannot be claimed. Future studies should focus on a longer period and conduct time series analysis to understand the effects of government policies and media reports on misinformation.

Nevertheless, those concurrences suggest that misinformation might not emerge randomly or evenly across time. Rather, misinformation of a topic may be induced by an event or information on the same topic. This is likely because ongoing events and information can act as circumstantial evidence for misinformation of a similar topic if they are not communicated effectively. This insight goes beyond the current research that predominantly examines when misinformation emerges (Rosnow, 1980, 1988), by suggesting how misinformation of a particular topic emerges. This suggestion is particularly critical for practitioners as it can strategize the allocations of limited communication resources for misinformation debunking. Future research should investigate how misinformation of a topic emerges and spreads along with ongoing events and information.

Additionally, as misinformation often emerge when official information is lacking (DiFonzo and Bordia, 2007), the findings suggest that crisis management policies, especially strong or extreme ones, should be supported by follow-up communication to ease the public from fear and uncertainty. News media should also frame their reports rigorously and scientifically to avoid misunderstandings.

This study has two limitations. First, the trend analysis was conducted with data of only one platform. As **Table 1** clearly shows that different fact-checking platforms tend to gather different themes of misinformation, future research should try to generalize the study results regarding the evolution of misinformation. Second, as this study utilized publicly available data on fact-checking platforms, it is unable to discover mechanisms why particular misinformation is made and circulated. Future studies should conduct surveys and experiments to understand how people create and spread misinformation during a disease crisis.

CONCLUSIONS

This study is the first to apply the CERC model to investigate the themes and evolution of misinformation during the early stages of an infectious disease outbreak. Though the study focused on misinformation that emerged surrounding COVID-19 in China, the findings are expected to be generalized into other public health emergencies because they are largely corresponding to the CERC model. This research is of theoretical and practical interest to communication scholars and practitioners who seek to maximize the effectiveness of outbreak communication by combating misinformation surrounding health crises. Future research should examine how and why misinformation is made and circulated by particular groups of people in specific crisis stages, to achieve successful crisis communication through combating misinformation.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

JL conceptualized the manuscript, analyzed the data, and contributed to the manuscript writing, reviewed the content, and agreed with submission. All authors contributed to the article and approved the submitted version.

REFERENCES

Bastani, P., and Bahrami, M. A. (2020). COVID-19 related misinformation on social media: a qualitative study from Iran. *J. Med. Internet Res*. doi: 10.2196/18932. [Epub ahead of print].

Bernard, H. R., Wutich, A., and Ryan, G. W. (2016). *Analyzing Qualitative Data: Systematic Approaches*. New York, NY: SAGE Publications.

Bessi, A., Coletto, M., Davidescu, G. A., Scala, A., Caldarelli, G., and Quattrociocchi, W. (2015). Science vs conspiracy: collective narratives in the age of misinformation. *PLoS ONE* 10:e0118093. doi: 10.1371/journal.pone.0118093

Brennen, J. S., Simon, F. M., Howard, P. N., and Nielsen, R. K. (2020). *Types, Sources, and Claims of Covid-19 Misinformation*. Reuters Institute. Available online at: http://www.primaonline.it/wp-content/uploads/2020/04/COVID-19_reuters.pdf (accessed June 26, 2020).

Centers for Disease Control and Prevention (2018). *Crisis and Emergency Risk Communication—2014 Edition*. Available online at: https://emergency.cdc.gov/cerc/manual/index.asp (accessed June 26, 2020).

Cohen, J. (2020). *Scientists "Strongly Condemn" Rumors and Conspiracy Theories About Origin of Coronavirus Outbreak*. Science Magazine. Available online at: https://www.sciencemag.org/news/2020/02/scientists-strongly-condemn-rumors-and-conspiracy-theories-about-origin-coronavirus (accessed June 26, 2020).

Cuan-Baltazar, J. Y., Muñoz-Perez, M. J., Robledo-Vega, C., Pérez-Zepeda, M. F., and Soto-Vega, E. (2020). Misinformation of COVID-19 on the internet: infodemiology study. *JMIR Public Health Surveill.* 6:e18444. doi: 10.2196/18444

DiFonzo, N., and Bordia, P. (2007). *Rumor Psychology: Social and Organizational Approaches*. Washington, DC: American Psychological Association.

Kouzy, R., Abi Jaoude, J., Kraitem, A., El Alam, M. B., Karam, B., Adib, E., et al. (2020). Coronavirus goes viral: quantifying the COVID-19 misinformation epidemic on Twitter. *Cureus* 12:e7255. doi: 10.7759/cureus.7255

Lazard, A. J., Scheinfeld, E., Bernhardt, J. M., Wilcox, G. B., and Suran, M. (2015). Detecting themes of public concern: a text mining analysis of the Centers for Disease Control and Prevention's Ebola live Twitter chat. *Am. J. Infect. Control* 43, 1109–1111. doi: 10.1016/j.ajic.2015.05.025

Lewandowsky, S., Stritzke, W. G., Freund, A. M., Oberauer, K., and Krueger, J. I. (2013). Misinformation, disinformation, and violent conflict: from Iraq and the "War on Terror" to future threats to peace. *Am. Psychol.* 68:487. doi: 10.1037/a0034515

Li, H. O., Bailey, A., Huynh, D., and Chan, J. (2020). YouTube as a source of information on COVID-19: a pandemic of misinformation?. *BMJ Glob. Health* 5:e002604. doi: 10.1136/bmjgh-2020-002604

Lwin, M. O., Lu, J., Sheldenkar, A., and Schulz, P. J. (2018). Strategic uses of Facebook in Zika outbreak communication: implications for the crisis and emergency risk communication model. *Int. J. Environ. Res. Public Health* 15:1974. doi: 10.3390/ijerph15091974

Oyeyemi, S. O., Gabarron, E., and Wynn, R. (2014). Ebola, Twitter, and misinformation: a dangerous combination?. *BMJ* 349:g6178. doi: 10.1136/bmj.g6178

Poland, G. A., and Spier, R. (2010). Fear, misinformation, and innumerates: how the Wakefield paper, the press, and advocacy groups damaged the public health. *Vaccine* 28:2361. doi: 10.1016/j.vaccine.2010.02.052

Reynolds, B., and Seeger, M. W. (2005). Crisis and emergency risk communication as an integrative model. *J. Health Commun.* 10, 43–55. doi: 10.1080/10810730590904571

Rosnow, R. L. (1980). Psychology of rumor reconsidered. *Psychol. Bull.* 87, 578–591. doi: 10.1037/0033-2909.87.3.578

Rosnow, R. L. (1988). Rumor as communication: a contextualist approach. *J. Commun.* 38, 12–28. doi: 10.1111/j.1460-2466.1988.tb02033.x

Rosnow, R. L. (1991). Inside rumor: a personal journey. *Am. Psychol.* 46:484. doi: 10.1037/0003-066X.46.5.484

Sommariva, S., Vamos, C., Mantzarlis, A., Đào, L. U., and Martinez Tyson, D. (2018). Spreading the (fake) news: exploring health messages on social media and the implications for health professionals using a case study. *Am. J. Health Educ.* 49, 246–255. doi: 10.1080/19325037.2018.1473178

Swire, B., and Ecker, U. (2018). "Misinformation and its correction: Cognitive mechanisms and recommendations for mass communication," in *Misinformation and Mass Audiences*, eds B. Southwell, E. A. Thorson, and L. Sheble (Austin, TX: University of Texas Press), 195–211.

Tandoc Jr, E. C., Lim, Z. W., and Ling, R. (2018). Defining "fake news": a typology of scholarly definitions. *Digit J.* 6, 137–153. doi: 10.1080/21670811.2017.1360143

Tu, Y., Qing, L., Yi, Z., and Xian, S. J. (2020). *People's Daily*. Available online at: https://news.sina.com.cn/c/2020-01-31/doc-iimxyqvy9378767.shtml?cre=tianyiandmod=pcpager_finandloc=16andr=9andrfunc=100andtj=noneandtr=9 (accessed June 26, 2020).

Vijaykumar, S., Nowak, G., Himelboim, I., and Jin, Y. (2018). Virtual Zika transmission after the first US case: who said what and how it spread on Twitter. *Am. J. Infect. Control* 46, 549–557. doi: 10.1016/j.ajic.2017.10.015

Waszak, P. M., Kasprzycka-Waszak, W., and Kubanek, A. (2018). The spread of medical fake news in social media–the pilot quantitative study. *Health Policy Tech.* 7, 115–118. doi: 10.1016/j.hlpt.2018.03.002

Wood, M. J. (2018). Propagating and debunking conspiracy theories on Twitter during the 2015–2016 Zika virus outbreak. *Cyberpsychol. Behav. Soc. Netw.* 21, 485–490. doi: 10.1089/cyber.2017.0669

Zhu, N., Zhang, D., Wang, W., Li, X., Yang, B., Song, J., et al. (2020). A novel coronavirus from patients with pneumonia in China, 2019. *N. Engl. J. Med.* 382, 727–733. doi: 10.1056/NEJMoa2001017

Conflict of Interest: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Medicine

BRIEF RESEARCH REPORT
published: 18 August 2020
doi: 10.3389/fmed.2020.00547

Viral Transmission and Clinical Features in Asymptomatic Carriers of SARS-CoV-2 in Wuhan, China

Fen Tan[1†], *Kaige Wang*[2†], *Jiasheng Liu*[3], *Dan Liu*[2*], *Jianfei Luo*[3*] *and Rui Zhou*[1*]

[1] Department of Pulmonary and Critical Care Medicine, The Second Xiangya Hospital, Respiratory Disease Research Institute of Hunan Province, Central South University, Changsha, China, [2] Department of Pulmonary and Critical Care Medicine, West China Hospital, Sichuan University, Chengdu, China, [3] Department of Gastrointestinal Surgery, Renmin Hospital of Wuhan University, Wuhan, China

We report the clinical characteristics, viral shedding duration, and contact tracing for asymptomatic carriers of SARS-CoV-2 in Wuhan, China. The asymptomatic carriers were relatively young (median age: 34.5 years). Chest computed tomography showed no abnormalities. The nasopharyngeal swab was an optimum specimen for RNA testing. The median viral shedding duration was 11.5 days. Notably, 2 months of viral shedding duration were reported in two nurses, which was much longer than previously reported or than usually thought. The transmissibility of SARS-CoV-2 by asymptomatic carriers during the studied period in Wuhan appeared to be weak. Only one patient (1/12) was found to have transmitted the virus to another person. Early asymptomatic carrier detection, isolation, and contact tracing could be useful to mitigate the spread of the disease.

Keywords: SARS-CoV-2, COVID-19, asymptomatic carrier, transmission ability, clinical features

OPEN ACCESS

Edited by:
Zisis Kozlakidis,
International Agency for Research on Cancer (IARC), France

Reviewed by:
Xinglou Yang,
Key Laboratory of Special Pathogens and Biosafety, Wuhan Institute of Virology (CAS), China
Xiaodong Zhang,
Jilin University, China

***Correspondence:**
Dan Liu
liudan10965@wchscu.cn
Jianfei Luo
afei099@163.com
Rui Zhou
zhourui2355@csu.edu.cn

†These authors have contributed equally to this work and share first authorship

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: *22 April 2020*
Accepted: *31 July 2020*
Published: *18 August 2020*

Citation:
Tan F, Wang K, Liu J, Liu D, Luo J and Zhou R (2020) Viral Transmission and Clinical Features in Asymptomatic Carriers of SARS-CoV-2 in Wuhan, China. Front. Med. 7:547. doi: 10.3389/fmed.2020.00547

INTRODUCTION

The epidemic of coronavirus disease 2019 (COVID-19) has spread globally and has resulted in more than 600,000 deaths as of late July (1). In the fight against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), an increasing number of investigators have begun to be focus on the risk of transmission from asymptomatic carriers. An asymptomatic carrier of SARS-CoV-2 is a person infected with SARS-CoV-2 who does not develop symptoms. According to their current disease state, asymptomatic carriers could be categorized as incubatory carriers in a pre-symptomatic state or convalescent carriers who have already recovered from the disease. Asymptomatic carriers play a critical role in the transmission of infectious diseases, including COVID-19 (2). The reported incidence of asymptomatic infections differs in various regions and time periods. An early report from China stated that only 1% of SARS-CoV-2 infections were asymptomatic (3). The incidence of asymptomatic infections on the "Diamond Princess" ship was 51.7% (4). A high proportion (40.7%) of asymptomatic infections was reported among residents and staff members of nine long-term care facilities in the USA (5). To date, a better understanding of asymptomatic carriers is still urgently needed. We hereby provide a report of healthy asymptomatic carriers based on a review and analysis of their medical records with the ultimate goal of mitigating the spread of COVID-19.

METHODS

We enrolled asymptomatic carriers of SARS-CoV-2 from March 20th, 2020, to April 5th, 2020, in the People's Hospital of Wuhan University. Of the 280 patients with laboratory-confirmed COVID-19, 12 patients who never developed any symptoms throughout the disease course were included in this study. The 12 asymptomatic carriers met the following criteria: (1). Confirmed SARS-CoV-2 infection, with patients having at least two positive results from RNA tests (6). Specimens collected from nasopharyngeal swabs, stool, and urine were analyzed by reverse transcription quantitative PCR to detect SARS-CoV-2 RNA; (2). A lack of related signs or symptoms of COVID-19, including fever and any respiratory symptoms during the entire hospitalization and the 14-day post-discharge isolation period. When SARS-CoV-2 infection was confirmed, the patients were hospitalized in the isolation ward until they met the discharge criteria. Discharge criteria was two consecutive negative results on RNA tests separated by at least a 24-h interval for nasopharyngeal swabs, stool, and urine. During hospitalization, all patients were treated with Arbidol (1,200 mg, three times per day, oral), and RNA testing was repeated every 3 days. After discharge, the patients were quarantined for 14 days in an isolated observation area and underwent viral RNA testing every week. When the test for viral RNA was confirmed to be positive in these patients, any person with whom the patients had contact was strictly quarantined for 14 days and tested for the presence of viral RNA. We analyzed all the clinical features, including the laboratory and radiographic findings. Data are presented as the medians ± interquartile ranges (IQRs) for continuous variables. The study was approved by the People's Hospital of Wuhan University Ethics Committee (No. WDRY2020-K068). Written informed consent was obtained from all the patients for the publication of any potentially identifiable data included in this article.

RESULTS

The proportion of patients from March 20th, 2020, to April 5th, 2020, in the People's Hospital of Wuhan University who were asymptomatic was 4.3%. The demographic and clinical characteristics of the 12 asymptomatic patients are shown in **Table 1**. All the patients were Wuhan residents. Seven patients were screened for viral RNA because of a definite history of exposure to confirmed patients, and two of the seven were nurses who had cared for COVID-19 patients in a front-line hospital. Four patients were screened for RNA when they were hospitalized due to an active disease, including acute pancreatitis, ectopic pregnancy, coronary heart disease, and hepatocellular carcinoma. One patient was screened for viral infection during a pre-employment physical examination and did not have a definite history of exposure. The patients had a median age of 34.5 years (IQR 29.0–43.0); nine of them were males and three of them were females.

Abbreviations: COVID-19, coronavirus disease 2019; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; IQR, interquartile range.

The retrospective results of RNA testing on specimens, including nasopharyngeal swabs, stool samples, and urine samples, during the entire illness duration are shown in **Table 1**. Nasopharyngeal swabs had the highest positive rates (12 of 12; 100%), followed by stool samples (6 of 12; 50%) and urine samples (1 of 12; 8.6%). Viral serological test results for IgM and IgG antibodies against SARS-CoV-2 are shown in **Table 1**. The IgG levels of all the patients were elevated (100%), and the IgM levels of two patients were slightly elevated (16.7%). Eight patients without active disease presented with normal laboratory markers, including white blood cell count and levels of C-reactive protein, lactate dehydrogenase, and D-dimer. Chest computed tomography was normal in all patients as well. Four patients who were hospitalized for active primary diseases had relevant changes in laboratory tests (**Table 1**). For example, both patient 1 and patient 7 presented with increased C-reactive protein levels because of acute pancreatitis and ectopic pregnancy surgery, respectively.

The duration from the first confirmed positive RNA test result to a confirmed negative RNA test result is defined as the viral shedding duration (7). The median shedding duration was 11.5 days (IQR 9.0–14.0), ranging from 7 to 70 days. Notably, the shedding duration in the two nurses (patient 8 and patient 9) was more than 2 months (**Table 1**), which was longer than previously reported or usually thought. We retrospectively tracked the disease course in these two nurses, and the details are shown in **Figure 1**. We found that these two nurses underwent viral RNA testing within 1 week after exposure to confirmed patients. The intervals from confirmed contact to the first RNA test in these two patients were 3 days and 6 days, which were much shorter than those in the other patients (**Table 1**). Additionally, patient 8 presented with recurrent positivity for viral RNA during the self-quarantine period after the first discharge. After performing contact tracing for all patients, only one patient was found to have transmitted the virus; he passed the virus to his mother, who developed mild COVID-19. Persons with whom the other 11 patients came into contact were not infected; either no symptoms were observed or screening viral RNA tests were negative during the 14-day isolation period.

DISCUSSION

SARS-CoV-2 is recognized as being much more transmissible than both SARS-CoV and Middle East respiratory syndrome coronavirus (8). Asymptomatic carriers of SARS-CoV-2 have received increasing attention (7, 9). In this report, we describe some asymptomatic carriers of the disease. Healthy asymptomatic carriers are more likely to be younger (median age: 34.5 years) than symptomatic hospitalized patients (median age: 56.0 years) (10), which is consistent with some recent studies (11). RNA tests of nasopharyngeal swabs were 100% positive, which is much higher than the positive rate in stool and urine specimens in asymptomatic carriers, suggesting that nasopharyngeal swabs could be the optimum samples. However, the nasopharyngeal swabs test was reported to deliver false negatives because of sample collection and the operating procedures in some studies

TABLE 1 | Summary of clinical features and laboratory findings.

	References	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8	Patient 9	Patient 10	Patient 11	Patient 12
Age	NA	35	43	45	30	27	22	32	34	29	42	52	64
Sex	NA	Male	Male	Male	Male	Male	Male	Female	Female	Female	Male	Male	Male
Occupation	NA	Office worker	Office worker	Office worker	Doctor	Office worker	Student	Office worker	Nurse	Nurse	Office worker	Office worker	Office worker
Contact history	NA	No	No	Yes	Yes	Yes	Yes	No	Yes	Yes	No	Yes	No
Transmission to contact	NA	No	No	No	No	No	Mother	No	No	No	No	No	No
Nasopharyngeal swabs	NA	+	+	+	+	+	+	+	+	+	+	+	+
Stool	NA	+	–	–	–	–	+	+	+	+	+	–	–
Urine	NA	–	–	–	–	–	+	–	–	–	–	–	–
Viral shedding duration	NA	11	8	25	11	11	12	7	70	65	9	14	14
Interval from contact to RNA testing	NA	NA	NA	46	20	24	14	NA	3	6	NA	57	NA
Comorbidity	NA	No	DM	No	No	No	No	No	No	No	CHD	No	HCC
Active diseases	NA	Acute pancreatitis	No	No	No	No	No	Ectopic pregnancy	No	No	CHD	No	HCC
WBC $\times 10^9$/L	3.5–9.5	6.1	7.8	6.34	7.8	7.29	7.0	12.97	4.37	4.49	6.92	8.09	2.18
Neutrophils $\times 10^9$/L	1.8–6.3	4.2	4.8	4.04	3.88	4.42	3.78	10.05	2.1	2.12	3.95	2.88	1.39
Lymphocytes $\times 10^9$/L	1.1–3.2	1.01	2.32	1.71	2.79	2.05	2.46	1.97	1.59	1.86	2.22	4.09	0.43
CRP, mg/L	<5	>200	<5	<5	<5	<5	<5	81	<5	<5	<5	<5	13
IgG*, AU/mL	<10	175.4	55.75	45.34	393.3	62.6	149.6	104	129	21.55	87.65	189.2	1,041
IgM*, AU/mL	<10	1.52	1.08	15.01	24.58	2.8	3.77	3.59	4.61	6.74	9.03	8.6	4.7
LDH, U/L	120–250	491	154	137	135	204	136	190	121	146	287	160	233
D-dimer, mg/L	0–0.55	9.54	0.21	0.14	0.21	0.1	0.27	0.8	0.15	NA	9.32	NA	NA

DM, diabetes mellitus; CHD, coronary heart disease; HCC, hepatocellular carcinoma; WBC, white blood cell; CRP, C-reactive protein; LDH, lactate dehydrogenase; NA, not available.

**IgG refers to antibody against SARS-CoV-2.*

**IgM refers to antibody against SARS-CoV-2.*

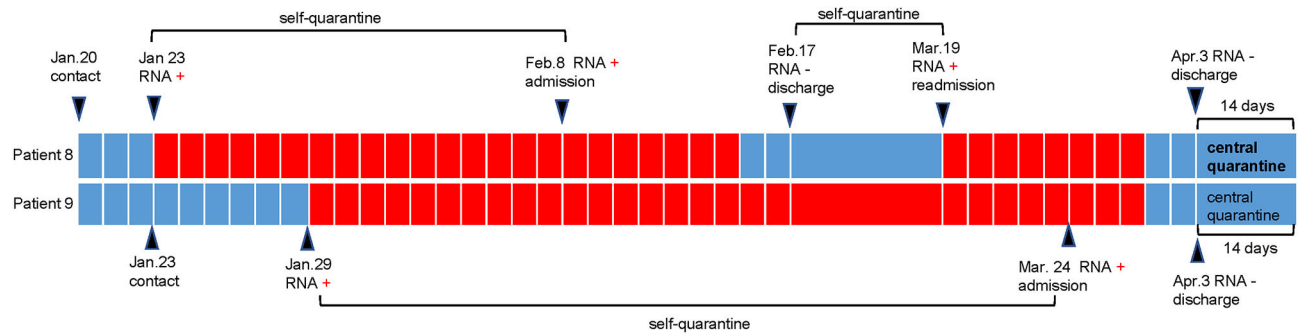


FIGURE 1 | Disease course in patient 8 and patient 9. As shown in the schematic, patient 8 and patient 9 (two nurses) performed their first RNA testing within 1 week after contact with the infected patient. Then they were self-quarantined until admission to hospital. During the period of self-quarantine and central quarantine, they were tested with nasopharyngeal swabs once a week. During the period of hospitalization, they were tested with nasopharyngeal swabs every 3 days. Patient 8 presented with recurrent positivity of RNA testing and was re-admitted to hospital.

(11). All asymptomatic carriers presented with elevated IgG antibody levels, while the IgM antibody levels were slightly elevated in a few patients in our study. The SARS-CoV-2 specific IgG and IgM yielded different responses depending on disease course. IgG usually maintains at high levels during a long period (12), while IgM usually wanes rapidly (13). IgG also reported to be seronegative in some asymptomatic individuals (7). Undoubtedly, the immune responses play a key role in the onset of COVID-2019. Hence, more immunological studies of asymptomatic carriers are needed urgently.

Prolonged viral shedding has been reported to be associated with fatal outcomes of severe influenza A (H7N9) infection (14). In COVID-19 non-survivors, the virus could be detected up until death. The viral shedding duration is a critical indicator of prognosis in symptomatic patients (10). It is estimated that viral shedding from asymptomatic carriers contributed to early transmission (15). In a previous retrospective cohort study, the median range of the duration of viral shedding among hospitalized patients was 12–20 days (10). The longest observed duration of viral shedding in survivors was 49 days (16). In study of 37 asymptomatic individuals (Chongqing, China), shedding duration was reported as 19 days (7). In our study, the median shedding duration of asymptomatic carriers was 11.5 days. Notably, the longest duration of viral shedding in two nurses was longer than 2 months. This is the longest viral shedding duration reported to date. When tracing the entire disease course, we found that the first RNA testing was performed much earlier in these two nurses than in other patients due to their confirmed exposure history. Besides early detection of RNA, the prolonged virus shedding duration in asymptomatic carriers is predicted to be associated with the frequency and quality of specimen collection (7). In addition, evidence of virus shedding duration only evaluated by reverse transcription quantitative PCR is limited, for RNA testing cannot distinguish whether the virus is alive or dead (17). The virus viability assessment in patients should be considered in future studies. One of the nurses developed a recurrent RNA positivity after discharge. Nasopharyngeal swabs, while effective, are unable to account for the possibility of reinfection and can also deliver false negative results (18). Our findings suggested that asymptomatic carriers could remain free from symptoms while carrying the virus for an extended period. This may provide evidence of the strong potential for transmission by asymptomatic carriers. Several studies have indicated that transmission of SARS-CoV-2 by asymptomatic carriers is implicated in crowds and family outbreaks in Wuhan, from December, 2019 to January, 2020 (19, 20). However, according to the results of contact tracing collected from March 20th, 2020, to April 5th, 2020 in our study, it was found that the transmissibility of the virus in asymptomatic carriers was weak, which is consistent with previous studies that suggested that the transmission risk is not high when patients are asymptomatic (21, 22). Additionally, it was reported that all 455 contacts who were exposed to the asymptomatic carriers in Guangdong, China, did not develop SARS-CoV-2 infection (23). Thus, the transmissibility of the virus in asymptomatic carriers might be weak. It is worth noting that the low transmissibility is probably related to the strict control measures implemented since February in Wuhan. More evidence is needed to clarify the transmissibility of the virus in asymptomatic carriers in the future. The collection of data from a larger cohort would enable researchers to more comprehensively investigate this issue.

In conclusion, viral RNA can be detected in asymptomatic carriers over a long period. The transmissibility of the virus in asymptomatic carriers from Wuhan, where strict control measures were implemented, was not as high as expected. Early asymptomatic carrier detection, isolation, and contact tracing would be useful to mitigate the spread of COVID-19. This report will hopefully provide a better understanding of the transmissibility of the virus in asymptomatic carriers of COVID-19.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

Written informed consent was obtained from the [individual(s) and/or minor(s)' legal guardian/next of kin] for the publication

of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

RZ, DL, and JLu directed the whole study to go on. KW, JLu, and JLi gathered information of all patients. FT and KW analyzed the data. FT drawed the table and the figure. FT wrote the manuscript. RZ, DL, and JLu reviewed and amended the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the National Key Clinical Specialty Constructions Project of China (Grant No. 2012650).

REFERENCES

1. Coronavirus disease. *(COVID-19) Situation Report – 188*. World Health Organization (2019).
2. Lai C-C, Liu YH, Wang C-Y, Wang Y-H, Hsueh S-C, Yen M-Y, et al. Asymptomatic carrier state, acute respiratory disease, and pneumonia due to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2): facts and myths. *J Microbiol Immunol Infect.* (2020) 53:404–12. doi: 10.1016/j.jmii.2020.02.012
3. Wu Z, McGoogan JM. Characteristics of and important lessons from the coronavirus disease 2019. (COVID-19) outbreak in China summary of a report of 72 314 cases from the Chinese center for disease control and prevention. *JAMA*. (2020). doi: 10.1001/jama.2020.2648. [Epub ahead of print].
4. Mizumoto K, Kagaya K, Zarebski A, Chowell G. Estimating the asymptomatic proportion of coronavirus disease 2019. (COVID-19) cases on board the Diamond Princess cruise ship, Yokohama, Japan, (2020). *Eurosurveillance.* (2020) 25:2000180. doi: 10.2807/1560-7917.ES.2020.25.10.2000180
5. Feaster M, Goh Y-Y. High proportion of asymptomatic SARS-CoV-2 infections in 9 long-term care facilities, Pasadena, California, USA, April 2020. *Emerg Infect Dis J.* (2020). doi: 10.3201/eid2610.202694. [Epub ahead of print].
6. Clinical protocols for the diagnosis and treatment of covid-19.Version 7. *National Health Commission and State Administration of Traditional Chinese Medicine.* (2020).
7. Long QX, Tang XJ, Shi QL, Li Q, Deng HJ, Yuan J, et al. Clinical and immunological assessment of asymptomatic SARS-CoV-2 infections. *Nat Med.* (2020). doi: 10.1038/s41591-020-0965-6. [Epub ahead of print].
8. Chen J. Pathogenicity and transmissibility of 2019-nCoV-A quick overview and comparison with other emerging viruses. *Microbes Infect.* (2020) 22:69–71. doi: 10.1016/j.micinf.2020.01.004
9. Yang R, Gui X, Xiong Y. Comparison of clinical characteristics of patients with asymptomatic vs symptomatic coronavirus disease 2019 in Wuhan, China. *JAMA Netw Open.* (2020) 3:e2010182. doi: 10.1001/jamanetworkopen.2020.10182
10. Zhou F, Yu T, Du R, Fan G, Liu Y, Liu Z, et al. Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: a retrospective cohort study. *Lancet.* (2020) 395:1054–62. doi: 10.1016/S0140-6736(20)30566-3
11. Tan J, Liu S, Zhuang L, Chen L, Dong M, Zhang J, et al. Transmission and clinical characteristics of asymptomatic patients with SARS-CoV-2 infection. *Future Virol.* (2020) doi: 10.2217/fvl-2020-0087. [Epub ahead of print].
12. Caturegli G, Materi J, Howard BM, Caturegli P. Clinical validity of serum antibodies to SARS-CoV-2: a case-control study. *Ann Intern Med.* (2020). doi: 10.7326/M20-2889. [Epub ahead of print].
13. Long QX, Liu BZ, Deng HJ, Wu GC, Deng K, Chen YK, et al. Antibody responses to SARS-CoV-2 in patients with COVID-19. *Nat Med.* (2020) 26:845–8. doi: 10.1038/s41591-020-0897-1
14. Wang Y, Guo Q, Yan Z, Zhou D, Zhang W, Zhou S, et al. Factors associated with prolonged viral shedding in patients with avian influenza A(H7N9) virus infection. *J Infect Dis.* (2018) 217:1708–17. doi: 10.1093/infdis/jiy115
15. Arons MM, Hatfield KM, Reddy SC, Kimball A, James A, Jacobs JR, et al. Presymptomatic SARS-CoV-2 infections and transmission in a skilled nursing facility. *N Engl J Med.* (2020) 382:2081–90. doi: 10.1056/NEJMoa2008457
16. Tan L, Kang X, Zhang B, Zheng S, Liu B, Yu T, et al. Plasma therapy cured a COVID-19 patient with long duration of viral shedding for 49 days: the clinical features, laboratory tests, plasma therapy and implications for public health management. *Med Comm.* (2020) 1:77–80. doi: 10.1002/mco2.2
17. Cangelosi GA, Meschke JS. Dead or alive: molecular assessment of microbial viability. *Appl Environ Microbiol.* (2014) 80:5884–91. doi: 10.1128/AEM.01763-14
18. Chen D, Xu W, Lei Z, Huang Z, Liu J, Gao Z, et al. Recurrence of positive SARS-CoV-2 RNA in COVID-19: a case report. *Int J Infect Dis.* (2020) 93:297–9. doi: 10.1016/j.ijid.2020.03.003
19. Chan JF, Yuan S, Kok KH, To KK, Chu H, Yang J, et al. A familial cluster of pneumonia associated with the 2019 novel coronavirus indicating person-to-person transmission: a study of a family cluster. *Lancet.* (2020) 395:514–23. doi: 10.1016/S0140-6736(20)30154-9
20. Bai Y, Yao L, Wei T, Tian F, Jin D-Y, Chen L, et al. Presumed Asymptomatic Carrier Transmission of COVID-19. *JAMA*. (2020) 323:1406–7. doi: 10.1001/jama.2020.2565
21. Zou L, Ruan F, Huang M, Liang L, Huang H, Hong Z, et al. SARS-CoV-2 viral load in upper respiratory specimens of infected patients. *N Engl J Med.* (2020) 382:1177–9. doi: 10.1056/NEJMc2001737
22. To KK-W, Tsang OT-Y, Leung W-S, Tam AR, Wu T-C, Lung DC, et al. Temporal profiles of viral load in posterior oropharyngeal saliva samples and serum antibody responses during infection by SARS-CoV-2: an observational cohort study. *Lancet Infect. Dis.* (2020) 20:565–74. doi: 10.1016/S1473-3099(20)30196-1
23. Gao M, Yang L, Chen X, Deng Y, Yang S, Xu H, et al. A study on infectivity of asymptomatic SARS-CoV-2 carriers. *Respir Med.* (2020) 169:106026. doi: 10.1016/j.rmed.2020.106026

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Public Health

ORIGINAL RESEARCH
published: 18 August 2020
doi: 10.3389/fpubh.2020.00486

Analysis of the Virus Contamination and Disinfection Effect in Isolation Ward of Patients With COVID-19

*Shiyang Zhang†, Chuanpeng Wang†, Minqiang Lin†, Qinsheng Deng, Yuzhen Ye, Zhiyong Li, Lixin Qiu and Zhanxiang Wang**

The First Affiliated Hospital of Xiamen University, Xiamen, China

OPEN ACCESS

Edited by:
Zisis Kozlakidis, International Agency for Research on Cancer (IARC), France

Reviewed by:
Devendra Tarachand Mourya, Indian Council of Medical Research (ICMR), India
Habeeb Kayode SAKA, Bayero University Kano, Nigeria
Xiaodong Gao, Zhongshan Hospital, Fudan University, China

***Correspondence:**
Zhanxiang Wang
sjwkwzx@163.com

†These authors have contributed equally to this work

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Public Health

Received: *17 March 2020*
Accepted: *30 July 2020*
Published: *18 August 2020*

Citation:
Zhang S, Wang C, Lin M, Deng Q, Ye Y, Li Z, Qiu L and Wang Z (2020) Analysis of the Virus Contamination and Disinfection Effect in Isolation Ward of Patients With COVID-19. Front. Public Health 8:486. doi: 10.3389/fpubh.2020.00486

The recent outbreak of COVID-19 has infected a large number of patients, increasing the importance of adequate disinfection of the hospital environment. We conducted this study to explore environmental virus contamination and the effect of terminal disinfection in the isolation ward of patients with COVID-19. A swab kit was used to sample various surfaces in the isolation and observation wards using the smear method. The samples were immediately sent to the PCR department of the laboratory for nucleic acid detection of COVID-19. We analyzed 31 high-frequency contact sites in three isolation wards of actively sick patients, of which seven were positive (22.58%, 7/31). Positive sites included the transfer window, bed rail, buffer room door handle, toilet door handle, and toilet faucet. All 55 samples taken from the wards of cured patients and the wards after terminal disinfection were negative. Virus contamination in areas frequently touched by patients in the isolation ward was high, so the awareness of correct disinfection must be increased. Use of 1,000–2,000 mg/L chlorine-containing disinfectant in the isolation ward was effective.

Keywords: COVID-19, environmental surface, virus contamination, cleaning and disinfection, hand hygiene

INTRODUCTION

Since the outbreak of COVID-19 in Wuhan, in December 2019 (1–3), more than 86,000 cases have been confirmed in China as of the end of 19 July 2020. The government have taken urgent measures, including declaring it a category B infectious disease and managing it as a category A infectious disease, the most stringent classification of infectious diseases (4). Suspected and confirmed cases of COVID-19 are diagnosed and treated in a specific hospital, which poses a challenge in terms of preventing cross-infection. The environment or objects in hospitals, once contaminated by patients' blood and body fluids (5), can be a new source of infection. If hands and clothing become contaminated, medical staff and other patients could be infected, causing hospital-acquired infections and even an infection outbreak. Therefore, it is important to effectively clean and disinfect environmental surfaces in hospitals (6, 7). To effectively guide disinfection of the hospital isolation ward, staff from the hospital infection management department went to the isolation ward of patients newly-diagnosed with COVID-19. Samples were taken from surfaces to investigate environmental virus contamination and the result of virus inactivation by the current disinfection method.

RESEARCH DESIGN AND METHODS

Research Design

We visited the designated COVID-19 treatment hospital in Xiamen, China from 25 February to 27 February 2020 and used the smear method to sample objects in the COVID-19 isolation wards. The sampling time was 2–4 h after disinfection, during which time patients moved about the ward freely. After the actively sick patients were transferred out of the ward, terminal disinfection was performed immediately. Further samples were taken 30 min after terminal disinfection was completed, to investigate whether the current disinfection method can eradicate all viruses on surfaces within the isolation ward of patients with COVID-19. Samples were also taken 2–4 h after disinfection from a ward of patients who had recovered from COVID-19.

Patient Diagnosis and Release Isolation Standard

According to the requirements of the COVID-19 Diagnosis and Treatment Program (Sixth Edition) (8), the respiratory specimens of patients were all positive for the detection of novel coronavirus nucleic acid by RT-PCR. The criteria for releasing the patients from the isolation are: 1. the patient's temperature is normal for more than 3 days; 2. respiratory symptoms have improved significantly; 3. pulmonary imaging shows significant improvement in exudative lesions in the acute phase; 4. Consecutive negative nucleic acid detection of respiratory specimens (across an interval of at least 24 h). To reduce the false-negative rate of the nucleic acid test, Xiamen hospital tests patients with a negative nucleic acid test three consecutive times across a sampling interval of at least 24 h, and then transfers them to the observation ward in the hospital for 14 days.

Sampling Method

A virus sampling swab kit was used in the isolation and observation wards to assess viral contamination in the environment. Following correct hand hygiene, we used virus sampling swabs to wipe the surface of places with high-frequency contact in the isolation ward (9, 10). The sampling area was 200–400 cm^2 on a broad surface, adjusted according to the size of the object surface, and the entire surface was wiped when it was <100 cm^2. The temperature in the room at the time of sampling was around 20°C. After sampling, the swab was put into sample storage solution and labeled. The specimens were sealed, the external surfaces disinfected and the sample was immediately transferred to the PCR room of the hospital laboratory for virus nucleic acid detection.

Environmental Cleaning and Disinfection Methods

The daily cleaning and disinfection method in the isolation ward is as follows: a cleaner wearing adequate personal protective equipment enters the isolation ward *via* a dedicated passage for medical staff. They use a 1,000–2,000 mg/L chlorine-containing disinfectant to wipe and disinfect the environmental surfaces at least twice a day. Air disinfection involves a plasma dynamic air disinfection machine, which absorbs and filters the dust in the air and also filters out microorganisms to reduce the risk of aerosol transmission of the coronavirus. Terminal disinfection was performed after patients were transferred out. This method involved an 80–120 mg/L hypochlorous acid spray for air disinfection. A dose of 10–20 ml/m^3 was atomized *via* a high-speed fan into tiny particles of <20 μm and sprayed evenly in the air to ensure it came into contact with microbial particles. A window was opened for ventilation after 60 min. Surfaces were wiped with a 1,000–2,000 mg/L chlorine-containing disinfectant. If textiles such as clothes or bed sheets needed to be reused, they were sterilized by circulating steam or boiling for 30 min; or soaked in 500 mg/L chlorine-containing disinfectant for 30 min, and then cleaned as usual. High-value textiles were cleaned using ethylene oxide.

TABLE 1 | Sampling results in isolation wards of actively sick patients with COVID-19.

Sampling site	Sample size	Positive sample	Positive rate
Transfer window	3	1	33.33%
Bed rail	3	1	33.33%
Bedside table	3	0	0.00%
Light switch	2	0	0.00%
Chair	1	1	100.00%
Floor	3	0	0.00%
Patient's washbasin	1	0	0.00%
Buffer room door handles	3	1	33.33%
Toilet door handles	3	1	33.33%
Toilet faucet	3	1	33.33%
Kettle	2	0	0.00%
Toilet surface	3	0	0.00%
Gloves after use	1	1	100.00%
Total	31	7	22.58%

Instruments and Reagents

Viral nucleic acid was detected using the new coronavirus nucleic acid detection kit (Xiamen Anpuli Biological Engineering Co., Ltd) as per the RT-PCR fluorescent probe method (11).

RESULTS

Environmental Surface Virus Contamination in the Isolation Ward of Actively Sick Patients With COVID-19

A total of 31 samples were collected from three isolation wards of patients actively sick with COVID-19. Of these samples, seven were positive (22.58%, 7/31). The positive specimens were sampled from the transfer window, bedrail, chair, one buffer room door handle, toilet door handles, toilet faucet and used gloves. The results are shown in **Table 1** and **Figure 1**.

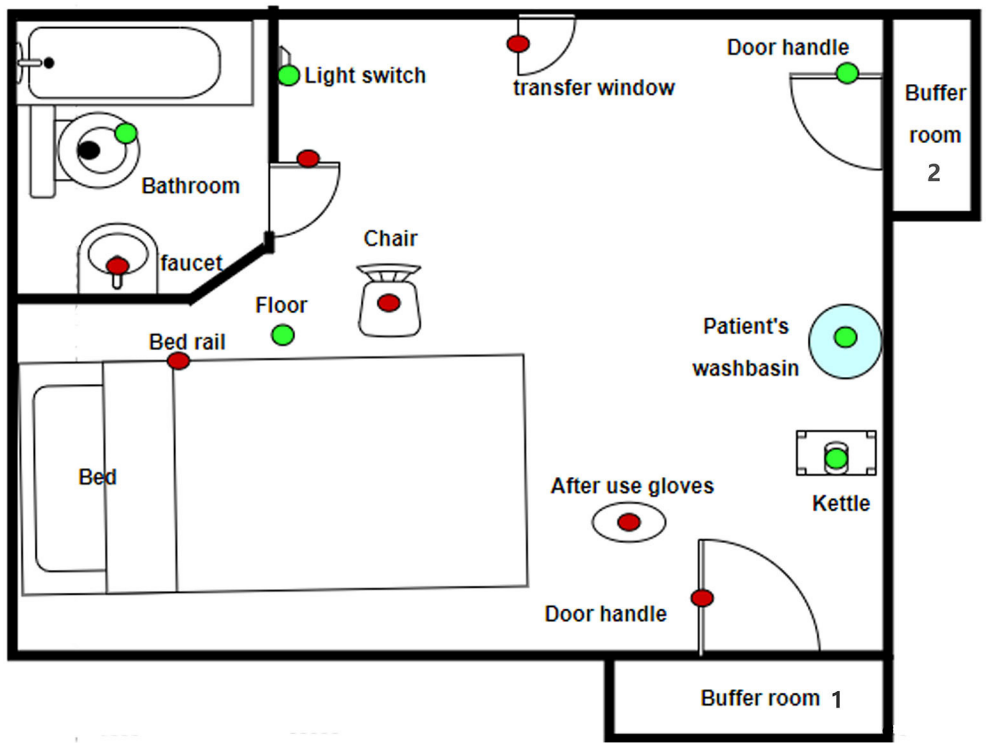


FIGURE 1 | Virus contamination in a typical isolation ward (green is negative, red is positive).

Environmental Surface Virus Contamination in the Isolation Ward of Cured Patients

A total of 31 samples were collected from three observation wards, including transfer windows, buffer room door handles, toilet faucets, bed rails, bedside tables, and so on. The samples were all negative for nucleic acid testing (**Table 2**), which means no virus was found in the ward of the cured patients.

Virus Inactivation After Terminal Disinfection in Isolation Ward of Actively Sick Patients of COVID-19

We sampled environmental surfaces in two isolation wards after terminal disinfection and obtained 24 samples, all of which were negative (**Table 2**). This means no virus contamination was found on environmental surfaces in the isolation ward after terminal disinfection.

DISCUSSION

Current research (8) shows that the main source of coronavirus infection is patients, including those who are asymptomatic, *via* droplet transmission and close contact. High concentrations of aerosols in relatively closed environments could lead to aerosol transmission (11, 12). According to research on coronavirus resistance, common disinfectants such as chlorine-containing disinfectants, 75% ethanol, hydrogen peroxide, and hypochlorous acid have good coronavirus inactivation effects (13, 14). Chlorine-containing disinfectants should be chosen if the objects are corrosion- resistant, but 75% ethanol should be chosen first when objects are non-resistant (15).

Patients with COVID-19 can directly contaminate surfaces in the surrounding environment through coughing and can contaminate their clothing or hands through improper cough etiquette. Contaminated clothing or hands can infect environmental surfaces. To guide the thorough infection of the surrounding environment, it was necessary to sample surfaces from an isolation ward of patients with COVID-19. Sampling was also performed to investigate the effect of terminal disinfection on virus inactivation.

A total of 31 samples were collected from the areas frequently contacted by actively sick patients, and the positive rate was 22.58% (7/31). The distribution of positive specimens was mainly on the toilet door handle, buffer room door handle, toilet faucet, and transfer window. Since these are all frequently touched, this indicates that the high-risk areas were those most likely to be contaminated through touching. This finding was supported by the positive sample collected from used gloves, indicating the higher risk of transmission *via* contaminated hands. In addition, the chairs and bed

TABLE 2 | Sampling results in isolation wards of cured patients with COVID-19 and the isolation wards after terminal disinfection.

Sampling site	Isolation ward of cured patients			Wards after terminal disinfection		
	Sample size	Positive sample	Rate	Sample size	Positive sample	Rate
Transfer window	2	0	0.00%	2	0	0.00%
Bed rail	2	0	0.00%	2	0	0.00%
Bedside table	3	0	0.00%	2	0	0.00%
Light switch	2	0	0.00%	1	0	0.00%
Chair	1	0	0.00%	2	0	0.00%
Floor	3	0	0.00%	2	0	0.00%
Buffer room door handles	3	0	0.00%	2	0	0.00%
Toilet door handles	3	0	0.00%	2	0	0.00%
Toilet faucet	3	0	0.00%	2	0	0.00%
Kettle	2	0	0.00%	1	0	0.00%
Toilet surface	3	0	0.00%	2	0	0.00%
Wall	1	0	0.00%	1	0	0.00%
Mattress	1	0	0.00%	1	0	0.00%
Equipment belt	1	0	0.00%	1	0	0.00%
Gloves After use	1	0	0.00%	1	0	0.00%
Total	31	0	0.00%	24	0	0.00%

rails may have been contaminated by the patient's clothing or hands.

The door handle of buffer room one was also positive. This was used by medical and cleaning staff to leave the ward and was barely touched by patients, indicating that not all medical staff or cleaners followed correct hand hygiene before leaving the ward. Therefore, we conducted hand hygiene training for all staff. Previous studies found similar results. Ong et al. (16) also demonstrated the survival of coronaviruses on the surfaces of a patient ward toilet and hand basin; Ye et al. (17) demonstrated the survival of coronaviruses on the surfaces of isolation ward door handles and used gloves; Kampf et al. (18) showed that the new coronavirus can survive on inanimate surfaces for a certain period of time (glass or plastic for up to 9 days). These studies show that contaminated objects may become a new source of infection, increasing the risk of cross-infection in the hospital.

Of the 31 specimens collected from three observation wards of recovered patients, no positive samples were found. This indicates that the hospital's protocol is successful. The protocol involves transferring the patient to an observation ward only after three consecutive negative nucleic acid tests performed 24 h apart and using 1,000–2,000 mg/L chlorine-containing disinfectant to wipe and disinfect the environmental surfaces. Similarly, samples were negative from the isolation ward after terminal disinfection, indicating that the current terminal disinfection method meets the requirements for virus inactivation. In order to reduce the risk of cross-infection in the hospital, 1,000–2,000 mg/L chlorine-containing disinfectant can be used for environmental cleaning and disinfection in short-term hospitalization, especially to strengthen the cleaning and disinfection of the high-frequency contact parts of the patients' hands.

There are certain shortcomings in this study, however. For example, as most cases have been cured, the sample size is limited and the contamination from patients with severe COVID-19 has not been investigated. No research has been conducted on other disinfectants and no research has been conducted on the disinfection effect of cloth. In the future, other regions or medical institutions with confirmed patients can explore this area further to provide more theoretical support to guide future disinfection strategies.

In summary, virus contamination in the isolation ward of actively sick patients with COVID-19 is common, especially in areas frequently touched by patients. Such high-frequency contact areas should be disinfected more frequently by enhancing the awareness of disinfection whenever necessary. The current terminal disinfection method of hypochlorous acid in the air combined with chlorine disinfectant on surfaces can meet the requirements of virus inactivation.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

This study had been reviewed and approved by the Ethics Committee of the First Affiliated Hospital of Xiamen University (Ethics Review Number 2020-003).

AUTHOR CONTRIBUTIONS

ZW and SZ conceived and designed the experiments. CW, ML, YY, ZL, and LQ performed the experiments. CW, QD, and SZ analyzed the data and wrote the manuscript. ZW and ML critically revised the manuscript. All authors read and approved the final version of the manuscript.

FUNDING

This work was supported by Xiamen Science and Technology Institution (3502Z2020YJ05) and the Chinese Geriatrics Society (GRYJ-KLB2018024).

ACKNOWLEDGMENTS

We also thank the patients and their families who supported this study.

REFERENCES

1. Zhai P, Ding YB, Wu X, Long JK, Zhong YJ, Li YM. The epidemiology, diagnosis and treatment of COVID-19. *Int J Antimicrob Agents*. (2020) 55:105955. doi: 10.1016/j.ijantimicag.2020.105955
2. Huang CL, Wang YM, Li XW, Ren LL, Zhao JP, Hu Y, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet*. (2020) 395:497–506. doi: 10.1016/S0140-6736(20)30183-5
3. Qin C, Zhou L, Hu Z, Zhang S, Yang S, Tao Y, et al. Dysregulation of immune response in patients with COVID-19 in Wuhan, China. *Clin Infect Dis*. (2020) 12:ciaa248. doi: 10.2139/ssrn.3541136
4. *National Health Commission of the People's Republic of China. Announcement of the National Health Committee of the People's Republic of China*. Available online at: http://www.nhc.gov.cn/jkj/s7916/202001/44a3b8245e8049d2837a4f27529cd386.shtml (accessed January 20, 2020).
5. Jiang YF, Wang HF, Chen YK, He JX, Chen LG, Liu Y, et al. Clinical data on hospital environmental hygiene monitoring and medical staffs protection during the coronavirus disease 2019 outbreak. *medRxiv*. (2020). doi: 10.1101/2020.02.25.20028043
6. Protano C, Cammalleri V, Spica Romano V, Valeriani F, Vitali M. Hospital environment as a reservoir for cross transmission: cleaning and disinfection procedures. *Ann Ig*. (2019) 31:436–48. doi: 10.7416/ai.2019.2305
7. Edmiston CE, Spencer M, Lewis BD, Rossi PJ, Brown KR, Malinowski M, et al. Assessment of a novel antimicrobial surface disinfectant on inert surfaces in the intensive care unit environment using ATP-bioluminesence assay. *Am J Infect Control*. 48:143–6. doi: 10.1016/j.ajic.2019.08.026
8. National Health Commission of the People's Republic of China. Diagnosis and treatment plan for pneumonitis caused by new coronavirus (trial version 6). *Chin J Nosocomiol*. (2020) 19:192–5. doi: 10.1097/CM9.0000000000000819
9. Rawlinson S, Ciric L, Cloutman-Green E. How to carry out microbiological sampling of healthcare environment surfaces? A review of current evidence. *J Hosp Infect*. (2019). 103:363–74. doi: 10.1016/j.jhin.2019.07.015
10. Hu XW, Xing YH, Ni W, Zhang F, Lu SY, Wang Z, et al. Environmental contamination by SARS-CoV-2 of an imported case during incubation period. *Sci Total Environ*. (2020) 742:140620. doi: 10.1016/j.scitotenv.2020.140620
11. Corman VM, Landt O, Kaiser M, Richard M, Adam M, Daniel KC, et al. Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. *Euro Surveill*. (2020) 25:2000045. doi: 10.2807/1560-7917.ES.2020.25.3.2000045
12. Hoehl S, Berger A, Kortenbusch M, Marhild K, Jindrich C, Denisa B, et al. Evidence of SARS-CoV-2 infection in returning travelers from Wuhan, China. *N Engl J Med*. (2020) 382:1278–80. doi: 10.1056/NEJMc2001899
13. Wei QH, Ren Z. Disinfection measures for new coronavirus-infected pneumonia epidemics in 2019. *Chin J Disinfect*. (2020) 37:59–62. doi: 10.11726/j.issn.1001-7658.2020.01.019
14. Zhang WF, He JM, Zhan JF. Coronavirus resistance and disinfection. *Chin J Disinfect*. (2020) 37:63–7. doi: 10.11726/j.issn.1001-7658.2020.01.020
15. Suo JJ, Yan ZQ, Liu YX, Chai GJ. Discussion on the status of nosocomial infection of new coronavirus pneumonia and its prevention and control strategies and measures. *Chinese J Nosocomiol*. (2020) 30:811–6. doi: 10.11816/cn.ni.2020-200231
16. Ong SWX, Tan YK, Chia PY, Lee TH, Ng OT, Yen MS, et al. Air, surface environmental, and personal protective equipment contamination by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) from a symptomatic patient. *JAMA*. (2020) 323:1610–2. doi: 10.1001/jama.2020.3227
17. Ye G, Lin H, Chen L, Wang S, Zeng ZK, Wang W, et al. Environmental contamination of SARS-CoV-2 in healthcare premises. *J Infect*. (2020) 81:e1–5. doi: 10.1016/j.jinf.2020.04.034
18. Kampf G, Todt D, Pfaender S, Steinmann E. Persistence of coronaviruses on inanimate surfaces and their inactivation with biocidal agents. *J Hosp Infect*. (2020) 104:246–51. doi: 10.1016/j.jhin.2020.01.022

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Medicine

OPINION
published: 18 August 2020
doi: 10.3389/fmed.2020.00522

COVID-19 Pandemic: Group Testing

Ozkan Ufuk Nalbantoglu[1,2] *and Aycan Gundogdu*[2,3]*

[1] Department of Computer Engineering, Erciyes University, Kayseri, Turkey, [2] Genome and Stem Cell Center (GenKok), Erciyes University, Kayseri, Turkey, [3] Department of Microbiology and Clinical Microbiology, Erciyes University, Kayseri, Turkey

Keywords: COVID-19, pandemia, molecular diagnostics, infection control, group testing

INTRODUCTION

The COVID-19 outbreak has revealed modern society to be negligently unprepared for a pandemic. Despite all the advancements in modern healthcare, our response has been that of a century ago. Back then, social distancing was the main mitigation strategy, and convalescent sera was the treatment option. In addition to that, today we have molecular (i.e., PCR) and serological (i.e., IgC/IgM) tests for diagnostics and ventilators for treatment. Given the pace of scientific and technological development in the last century, this pattern implies the lack of translating scientific knowledge to applications.

Leaving the therapeutics out of scope, we would like to comment on the diagnostics perspective from the early stages: infection control via preventive diagnostics. The most natural question to ask is "Was the pandemic inevitable?" This is a tough question to answer. There are strong arguments supporting both answers. On one hand, epidemiological simulations considering the contemporary aviation schedules have arrived at a significant conclusion: it is highly probable that an outbreak of such parameters would have ended up evolving into a pandemic (1). It is indeed hard to reject the notion that SARS-CoV-2, with its relatively long transmissible incubation period, could easily travel and cover the human habitat, and it would be impossible to trace with conventional measures. The mainstream approach to infection control during the global spread has been scanning potential carriers using symptomatic signals (e.g., thermal cameras, thermometer checks, travel questionnaires, etc.) at connecting hubs. However, these preventative measures were shown to be ineffective in eliminating the spread of COVID-19. The main factor why the preventive measures fell short might be the appearance of excessive numbers of asymptomatic/presymptomatic carriers, which are difficult to detect. An unbiased estimation of the ratio of asymptomatic or presymptomatic spreaders might be difficult to assess. Statistics from small- or medium-sized cohorts and case studies indicate that they might be as abundant as 10–50% of the total number of infections (2, 3). These ratios hold special importance regarding the case studies, showing that asymptomatic/presymptomatic carriers are likely to infect their contacts (4, 5).

The counter-argument claiming that a pandemic in the contemporary world would have been preventable relies on an extensive use of modern digital technology. The idea is a working communication infrastructure as an early warning system. It is believed that such a system could enable control of the epidemic at initial phases. This optimism perhaps stems from the early detection success of The Global Public Health Intelligence Network (GPHIN) (6) during the first SARS-CoV-1 outbreak. Previously, it was believed that utilizing cellular networks would be an invaluable non-biotechnological opportunity for early detection and response. Note that this was even before the emergence of mobile technologies, big data, widespread social networks, and the tremendous advances in artificial intelligence fields. The last decade had been a time of blooming opinions and futuristic depictions of how technology and society is transforming into a new and data-driven paradigm. Shifting from the diagnostic care of twentieth century to the preventive strategies of twenty-first century for emerging infectious diseases was obviously no exception. It was expected that big data analytics could be the key to rapid detection and early prevention of the next pandemic (7–9).

OPEN ACCESS

Edited by:
Alexander Rodriguez-Palacios, Case Western Reserve University, United States

Reviewed by:
Endre Csóka, Alfred Renyi Institute of Mathematics (MTA), Hungary
Abraham S. Alabi, Centre de Recherche Médicales de Lambaréné, Gabon

***Correspondence:**
Aycan Gundogdu
agundogdu@erciyes.edu.tr

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: *14 April 2020*
Accepted: *27 July 2020*
Published: *18 August 2020*

Citation:
Nalbantoglu OU and Gundogdu A (2020) COVID-19 Pandemic: Group Testing. Front. Med. 7:522. doi: 10.3389/fmed.2020.00522

DIGITAL TECHNOLOGY AND DATA SCIENCE IN EPIDEMIOLOGY

As of February 2020, almost the half of the world voluntarily carries GPS tracking devices (i.e., smartphones), which can record the mobility of masses[1]. A vast majority of the transmission from ground zero, patient one, and day 1 to the current terminal points sits unexplored in web servers both as transmission networks and as spatial distributions. Nevertheless, falling short of early detection, the technology field has reacted with a great effort to fight against the pandemic. In fact, an artificial intelligence (AI)-backed outbreak risk estimator warned of the Wuhan outbreak, preparing even before the WHO and CDC (10). Mobile tracking of possible infections has been extensively used, firstly by South Korea and Singapore (11), and it has rapidly become a widespread technological help used by several nations (12). In addition, the use of data science and big data analytics from rich information sources appears to be on track. Simple digital surveys to locate infection clusters (13) and monitoring surveillance using online data sources (14) were adopted as common practices. Diagnostics using AI-backed biomedical signal processing on medical imaging emerged with practical applications. Deep learning on computerized tomography scans aims to remove the burden on the physicians overwhelmed by the explosion of cases (14, 15). Digital technology was not enough alone to prevent a pandemic; however, it is plausible that it is transforming into strong tools with which to fight and perhaps mitigate it. It might not be possible to conclude whether the state of the art can prevent or control pandemics in the mist of crisis, and there is little data yet to prove this. However, it is agreed that digital technology and data science should be destined to be an integral part of epidemiology in post-COVID-19 practice (16).

MOLECULAR DIAGNOSIS OF COVID-19

Besides being caught short in several fields from public health measures to digital technology, we advocate the claim that the greatest aforementioned scientific translation is in the field of molecular diagnostics. The popular view sets a premium on testing, assessing which is the single most effective weapon with which to track, explore, and isolate the transmission clusters[2]. In fact, testing strategies of different nations have interestingly validated the importance of testing as a preventative diagnostics strategy. The supporting data assessed by epidemic curve characteristics showed the effectiveness of mass testing regimes (17). Motivated by the revealing data, public health decision makers all around the world are trying to switch to extensive testing setups in order to reduce the infection transmission as much as possible. At this point, it is worth questioning the testing routine adopted by the global community. The mainstream molecular method of COVID-19 diagnosis is PCR-based amplicon detection (RT-qPCR in a practical set up) of SARS-CoV-2 genetic material. With widespread infrastructure and routine experience, this seems to be a natural and feasible solution. However, considering the capacity and current scalability options of PCR testing, it is uncertain whether this is the "extensive testing" scenario we are dealing with. By early April 2020, the total amount of tests conducted were in the millions band[3]. The United States, performing the greatest number of daily tests with more than 100,000 tests/day[4], is now seeing a surge in testing capacity. As the epidemic curve is steepened, it is very likely that the current regime is underperforming. While the current approach is a peacetime (i.e., endemic dynamics) convention, we are in wartime (i.e., pandemic dynamics), which requires its own unique measures.

Along with the technical scalibility issues of current testing conventions, it should also be taken into consideration that waiting to initiate and ramp up the testing availability contributes to the development of steep epidemic curves. These factors are heavily reliant on the differing response policies of governments (18, 19), complex legal oversights for the eligibility to test (20), and technical and economical unpreparedness, especially in third-world countries (21). Regardless of the state of the art molecular testing, the related social issues would have been a significant obstacle to employ extensive testing.

HOW FAR CAN WE GO IN TESTING WITH THE AVAILABLE RESOURCES AND WHAT WOULD WE FACE?

Outbreak simulations imply that even an imperfect detection and isolation at population levels might be enough to control the COVID-19 outbreak (22). It is difficult to assess whether there are stability breakpoints after which social isolation remains the single most effective measure to reduce risk of spread. However, it can be hypothesized that widespread scale testing—enough to trace more than 70% of contacts—at early arrival phases will be very effective in controlling the outbreaks. Considering that the first wave of outbreaks might have not hit certain societies, and resurgence will still be a great risk globally; more aggressive large-scale testing techniques need to be a priority of molecular microbiology. Furthermore, even in the late epidemic phases, large-scale testing would be a dampening factor flattening the epidemic curves.

As per wartime resources, we do not refer to novel molecular techniques or groundbreaking early-level technologies but very common conventions: RT-qPCR and next-generation sequencing. It could be possible to scale up the testing capacity at orders of magnitude, introducing only simple procedures on well-known daily lab routines. Firstly, considering that the popular biotechnological subject of the last 15 years has abruptly disappeared from the radar, the scientific society had

[1]https://www.statista.com/statistics/330695/number-of-smartphone-users-worldwide/

[2]https://www.who.int/docs/default-source/coronaviruse/transcripts/who-transcript-emergencies-coronavirus-press-conference-full13mar2020848c48d2065143bd8d07a1647c863d6b.pdf?sfvrsn=23dd0b04__2

[3]https://ourworldindata.org/covid-testing

[4]https://covidtracking.com/data/

been praising the high-throughput capability of next-generation sequencing. To date, the attempts to use NGS have been mainly on the sequence analysis of SARS-CoV-2[5] (23, 24), and it has not become a common procedure of testing. The underlying reason for this might be the fact that multiplexing and barcoding preferences are not designed for extreme sample numbers. However, theoretically, a single Illumina sequencer can, for example, cover the SARS-CoV-2 genome 12 billion times in a 24-h run[6]; hundreds of thousands if not millions of samples could be tested in a single spot. That could sound like overestimation, neglecting several practical limitations, but feasible proposals with impressive capacity offerings exist[7]. Released the protocols for a massively parallel COVID-19 diagnostic assay enabling simultaneous testing of 19,200 patient samples. The suggested assay includes a clever tweak in which a large number of barcodes are integrated to a reverse transcription step that enables large-scale testing in a single PCR and sequencing run. It is possible to design multitudes of such laboratory procedures that numerous NGS laboratories are capable of adopting and applying in the blink of an eye. Transferring NGS superpower to the COVID-19 testing arsenal would not only remove the burden from veteran and surging PCR technology, but it would also bring the possibility of mass testing one step closer.

POPULATION LEVEL SCANNING FOR COVID-19

A second opportunity we have been overlooking is not as visible as high-throughput sequencing, but it is an old, well-known wartime tussle invented to exploit limited resources: group testing. Back in the 1940s, the need for screening US army recruits for syphilis arose. As collecting blood samples and performing a single Wassermann test for each man appeared to be quite resource demanding in the circumstances of World War II, pooling blood samples and performing group tests was observed to be quite effective since the disease was relatively rare. Later on, group testing has become a popular topic in the information theory field, enabling orders of magnitude saving from the test numbers while being able to pinpoint sparse positives accurately (25). Similarly, the attractiveness of recovering sparse signals from a small number of measurements led, in the mid-2000s, to the birth of an entire research area called compressive sampling (compressed sensing) in the signal processing field around (26). Compressive sampling ideas converge into group testing for special settings where sampling matrices are binary (pooling) designs. Several theoretical results (27) and practical applications (28) have been reported, and, from a computational point of view, it can be annotated as a mature field. There have been few studies investigating the group testing opportunities in genotyping (29), and it was not a major point of attraction for molecular diagnostics, perhaps because demand was not particularly high. The notion of "a single specimen per test reaction" is now by default synonymous with diagnostic testing. On the other hand, compressing a very large number of tests in random/structured pools (e.g., around 40 to 120 samples per pooling tube) and conducting relatively small numbers of group tests that are decodable to original results is a tempting idea for the purpose of allocating resources efficiently. There are convincing preliminary results showing that pooling samples in feasible ranges would not attenuate the positive signals to undetectable levels in RT-qPCR (30). Similarly, given enough sequencing depth, detectability could be conserved in NGS testing. In fact, elemental ideas of simple group testing are blooming[8] (31–33). Sinnott-Armstrong et al. (28) proposed that a simple grouping scheme pooling on rows and columns of well-plates could increase the testing rates to around 4.5- and 7-fold for 96-well plate and 384-well plate applications, respectively, for 1% prevalence of positive cases. This scheme could achieve up to 9.5-fold increase (384-well plate setting) at a testing rate of 0.1% prevalence of positive cases. It should be acknowledged that this valuable boost does not explore the theoretical and practically achievable rates of compressive sampling capabilities. While theoretically perfect, reconstruction of original test results available with not much more than $k \log_2(N/k)$ measurements (25), where N is the number of samples and k is the number of positive cases, with the use of modern decoding algorithms, the achievable rates are close to the theoretical bounds. This means a 10- to 20-fold rate increase for a 1–0.1% prevalence band is possible with more sophisticated pooling schemes and decoding algorithms. In fact, allowing for more than one round of testing, namely, adaptive testing, instead of one-shot recovery of results, can provide even more efficient outcomes. Especially for low prevalence regimes (i.e., $P < 1/K^2$), N $(2P + (1\text{-}2P)/K)$ measurements set a lower bound on the number of required tests, where P is the prevalence and K the limit of the pool size (34). This implies almost a couple of tests per a positive sample and a single test per pool—a very efficient scheme with large pools. Recently, Shental et al. (35) sampled 48 pools out of a 384 well-plate by way of Reed-Solomon coding and showed an 8X efficiency gain around the band of 1% prevalence in a realistic laboratory setting. In fact, simulations showed that up to 60X expansion in testing capacity is available at around 2–3% of the prevalence band (36). This result might be an implication that large-scale contact tracing might be possible at early forming clusters. A further fascinating result we can draw from the compressive sampling field is that, as the number of samples increase and the prevalence decreases, the sampling efficiency scales up to impressive rates. This phenomenon would result in ultra-throughput testing with a moderate number of actual tests. For example, for the case of sudocodes, at a prevalence of 0.1%, 1 million subjects can be scanned by performing under 10,000 tests (37). We can assume that this scenario realistically fits into the population level testing ambition, in case of early arrival of the pandemic curve. The possible scenarios

[5]https://nanoporetech.com/about-us/news/uk-creates-covid-19-genome-sequencing-alliance-large-scale-analysis-virus-oxford

[6]https://www.illumina.com/content/dam/illumina-marketing/documents/products/datasheets/novaseq-6000-system-specification-sheet-770-2016-025.pdf

[7]https://docs.google.com/document/d/1kP2w__uTMSep2UxTCOnUhh1TMCjWvHEY0sUUpkJHPYV4

[8]https://www.tse-fr.eu/sites/default/files/TSE/documents/doc/by/gollier/group_testing.pdf

might be either as extending contact tracing to be able to test greater number of case contacts or as a periodic scan of specific populations such as scans at the level of family, school, classroom, workplace/office, daycare, healthcare workers, and other at-risk groups, staying within the available testing budgets. The opportunity for periodic economical scanning of specific groups could be operationalized as a powerful security measure in the phase of reopening economies. Taking the NGS recruit discussion above into consideration (i.e., tens of thousands of tests can be run on a single sequencer with a single PCR reaction), it can even be proposed that scanning of a million subjects could potentially be conducted in one diagnostic center in a single shot. Of course, this assessment neglects the enormous swab sampling, logistics, and sample preparation aspects. Our sole claim here, however, is that, with the modern molecular diagnostics technology, population level scanning should not be a real bottleneck in outbreak control.

CONCLUSION

The COVID-19 pandemic has caught modern society unprepared. Imposed outbreak measures have fallen short of mitigating the evolution of the outbreak into a pandemic. In this opinion article, we discussed whether infection control via preventive diagnostics could be a strong tool in our fight. Currently, digital technology and data science are becoming integral tools with which to help in the control of outbreaks. Although there have been great advancements in molecular technologies, there seems to be a lack of scientific translation in molecular diagnostics. With its surging capacity, RT-qPCR use in COVID-19 diagnosis is underperforming when conducting population-level scans. Despite its grand potential in high-throughput diagnosis, the next-generation sequencing systems have not been deployed sufficiently. Moreover, advanced algorithms to conduct group testing could enable large-scale testing for detecting and isolating infection clusters. Therefore, the scientific community should seek ways to translate invaluable technical expertise to fighting the COVID-19 pandemic, and it should also seek to integrate next-generation tools to contemporary practice. Testing *en masse* might not be as infeasible as it is confined to limited ideas and practices. The availability of detecting and isolating emerging clusters, thus minimizing the infection contacts, could pave the road to avoiding nation-level lockdowns and undetermined periods of quarantine measures. Otherwise, relying on only social distancing will be nothing but failing the "test."

AUTHOR CONTRIBUTIONS

All authors listed have equally made a substantial, direct and intellectual contribution to the work, and approved it for publication.

REFERENCES

1. Sato AH, Ito I, Sawai H, Iwata K. An epidemic simulation with a delayed stochastic SIR model based on international socioeconomic-technological databases. In *2015 IEEE International Conference on Big Data (Big Data)*. Santa Clara, CA: IEEE (2015). p. 2732–41.
2. Mizumoto K, Kagaya K, Zarebski A, Chowell G. Estimating the asymptomatic proportion of coronavirus disease 2019 (COVID-19) cases on board the Diamond Princess cruise ship, Yokohama, Japan, 2020. *Euro Surveill.* (2020) 25:2000180. doi: 10.2807/1560-7917.ES.2020.25.10.2000180
3. Nishiura H, Kobayashi T, Miyama T, Suzuki A, Jung S, Hayashi K, et al. Estimation of the asymptomatic ratio of novel coronavirus infections (COVID-19). *Int J Infect Dis.* (2020) 94:154–5. doi: 10.1016/j.ijid.2020.03.020
4. Bai Y, Yao L, Wei T, Tian F, Jin DY, Chen L, et al. Presumed asymptomatic carrier transmission of COVID-19. *JAMA*. (2020) 323:1406–7. doi: 10.1001/jama.2020.2565
5. Gandhi M, Yokoe DS, Havlir DV. Asymptomatic transmission, the Achilles' heel of current strategies to control COVID-19. *N Engl J Med.* (2020) 382:2158–60. doi: 10.1056/NEJMe2009758
6. Mykhalovskiy E, Weir L. The global public health intelligence network and early warning outbreak detection. *Can J Public Health.* (2006) 97:42–4. doi: 10.1007/BF03405213
7. Astill J, Dara RA, Fraser ED, Sharif S. Detecting and predicting emerging disease in poultry with the implementation of new technologies and big data: a focus on Avian Influenza Virus. *Front Vet Sci.* (2018) 5:263. doi: 10.3389/fvets.2018.00263
8. USAID (U.S. Agency for International Development). *Emerging Pandemic Threats*. (2016). Available online at: https://www.usaid.gov/news-information/fact-sheets/emerging-pandemic-threats-program (accessed June 2, 2016).
9. Dion M, Abdel Malik P, Mawudeku A. Big data: big data and the global public health intelligence network (GPHIN). *Can Commun Dis Rep.* (2015) 41:209. doi: 10.14745/ccdr.v41i09a02
10. Inn TL. *Smart City Technologies Take on COVID-19*. Penang: World Health (2020).
11. Zastrow M. South Korea is reporting intimate details of COVID-19 cases: has it helped? *Nature.* (2020). doi: 10.1038/d41586-020-00740-y. [Epub ahead of print].
12. Ienca M, Vayena E. On the responsible use of digital data to tackle the COVID-19 pandemic. *Nat Med.* (2020) 26:463–4. doi: 10.1038/s41591-020-0832-5
13. Rossman H, Keshet A, Shilo S. A framework for identifying regional outbreak and spread of COVID-19 from one-minute population-wide surveys. *Nat Med.* (2020) 26:634–8. doi: 10.1038/s41591-020-0857-9
14. Ting DSW, Carin L, Dzau V, Wong TY. Digital technology and COVID-19. *Nat Med.* (2020) 26:459–61. doi: 10.1038/s41591-020-0824-5
15. Shan F, Gao Y, Wang J, Shi W, Shi N, Han M, et al. Lung Infection Quantification of COVID-19 in CT Images with Deep Learning. *arXiv preprint* arXiv:2003.04655 (2020).
16. Gozes O, Frid-Adar M, Greenspan H, Browning PD, Zhang H, Ji W, et al. Rapid AI development cycle for the coronavirus (covid-19) pandemic: initial results for automated detection & patient monitoring using deep learning CT image analysis. *arXiv preprint* arXiv:2003.05037 (2020).
17. Balilla J. *Assessment of COVID-19 Mass Testing: The Case of South Korea* (2020). Available online at: https://ssrn.com/abstract=3556346
18. Carter, DP, May, PJ. Making sense of the US COVID-19 pandemic response: a policy regime perspective. *Admin TheorPraxis.* (2020) 42:265–77. doi: 10.1080/10841806.2020.1758991
19. Shvetsova O, Catalano M, Chu H, Dumond GK, Muftuoglu E, Ozutemiz H, et al. *Policy Error and Policy Rescue in COVID-19 Responses in the United States and United Kingdom* (2020). Available online at: https://orb.binghamton.edu/working_paper_series/3/
20. McDermott A. Inner Workings: molecular biologists offer "wartime service" in the effort to test for COVID-19. *Proc Natl Acad Sci USA.* (2020) 117:9656–9. doi: 10.1073/pnas.2006240117
21. Gilbert M, Pullano G, Pinotti F, Valdano E, Poletto C, Boëlle P-Y, et al. Preparedness and vulnerability of African countries against

importations of COVID-19: a modelling study. *Lancet*. (2020) 395:871–7. doi: 10.1016/S0140-6736(20)30411-6
22. Hellewell J, Abbott S, Gimma A, Bosse NI, Jarvis CI, Russell TW, et al. Feasibility of controlling COVID-19 outbreaks by isolation of cases and contacts. *Lancet Glob Health*. (2020) 8:e488–96. doi: 10.1016/S2214-109X(20)30074-7
23. Andersen KG, Rambaut A, Lipkin WI, Holmes EC, Garry RF. The proximal origin of SARS-CoV-2. *Nat Med*. (2020) 26:450–2. doi: 10.1038/s41591-020-0820-9
24. Sah R, Rodriguez-Morales AJ, Jha R, Chu DKW, Gu H, Peiris M, et al. Complete genome sequence of a 2019 novel coronavirus (SARS-CoV-2) strain isolated in Nepal. *Microbiol Resour Announc*. (2020) 9:e00169-20. doi: 10.1128/MRA.00169-20
25. Aldridge M, Johnson O, Scarlett J. Group testing: an information theory perspective. *Found Trends Commun Inform Theor*. (2019) 15:196–392. doi: 10.1561/0100000099
26. Candès EJ, Wakin MB. An introduction to compressive sampling. *IEEE Signal Process Magaz*. (2008) 25:21–30. doi: 10.1109/MSP.2007.914731
27. Atia GK, Saligrama V. Boolean compressed sensing and noisy group testing. *IEEE Trans Inform Theor*. (2012) 58:1880–901. doi: 10.1109/TIT.2011.2178156
28. Sinnott-Armstrong N, Klein D, Hickey B. Evaluation of group testing for SARS-CoV-2 RNA. *medRxiv [Preprint]*. (2020). doi: 10.1101/2020.03.27.20043968
29. Erlich Y, Gordon A, Brand M, Hannon GJ, Mitra PP. Compressed genotyping. *IEEE Trans Inform Theor*. (2010) 56:706–23. doi: 10.1109/TIT.2009.2037043
30. Yelin I, Aharony N, Shaer Tamar E, Argoetti A, Messer E, et al. Evaluation of COVID-19 RT-qPCR test in multi-sample pools. *Clin Infect Dis*. (2020). doi: 10.1093/cid/ciaa531. [Epub ahead of print].
31. Shani-Narkiss H, Gilday OD, Yayon N, Landau ID. Efficient and practical sample pooling high-throughput PCR diagnosis of COVID-19. *medRxiv [Preprint]*. (2020). doi: 10.1101/2020.04.06.20052159
32. de Wolff T, Pflüger D, Rehme M, Heuer J, Bittner MI. Evaluation of pool-based testing approaches to enable population-wide screening for COVID-19. *arXiv preprint* arXiv:2004.11851 (2020).
33. Eberhardt JN, Breuckmann NP, Eberhardt CS. Multi-stage group testing improves efficiency of large-scale COVID-19 screening. *J Clin Virol*. (2020) 128:104382. doi: 10.1016/j.jcv.2020.104382
34. Csóka E. Application-oriented mathematical algorithms for group testing. *arXiv preprint* arXiv:2005.02388 (2020).
35. Shental N, Levy S, Skorniakov S, Wuvshet V, Shemer-Avni Y, Porgador A, et al. Efficient high throughputSARS-CoV-2 testing to detect asymptomatic carriers. *medRxiv [Preprint]*. (2020). doi: 10.1101/2020.04.14.20064618
36. Nalbantoglu O. Group testing performance evaluation for SARS-CoV-2 massive scale screening and testing. *BMC Med Res Methodol*. (2020) 20:176. doi: 10.1186/s12874-020-01048-1
37. Sarvotham S, Baron D, Baraniuk RG. Sudocodes-fast measurement and reconstruction of sparse signals. In: *2006 IEEE International Symposium on Information Theory*. Seattle, WA: IEEE (2006). p. 2804–8.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Artificial Intelligence

MINI REVIEW
published: 18 August 2020
doi: 10.3389/frai.2020.00065

Artificial Intelligence for COVID-19 Drug Discovery and Vaccine Development

*Arash Keshavarzi Arshadi[1], Julia Webb[1], Milad Salem[2], Emmanuel Cruz[3], Stacie Calad-Thomson[4], Niloofar Ghadirian[5], Jennifer Collins[1], Elena Diez-Cecilia[3], Brendan Kelly[3], Hani Goodarzi[6] and Jiann Shiun Yuan[2]**

[1] Burnett School of Biomedical Sciences, University of Central Florida, Orlando, FL, United States, [2] Department of Electrical and Computer Engineering, University of Central Florida, Orlando, FL, United States, [3] A2A Pharmaceuticals, Cambridge, MA, United States, [4] Atomwise Inc., San Francisco, CA, United States, [5] Department of Chemistry and Biochemistry, University of Arizona, Tucson, AZ, United States, [6] Department of Biochemistry and Biophysics, Helen Diller Family Comprehensive Cancer Center, University of California, San Francisco, San Francisco, CA, United States

OPEN ACCESS

Edited by:
Weida Tong,
National Center for Toxicological Research (FDA), United States

Reviewed by:
Xiaowei Xu,
University of Arkansas at Little Rock, United States
Zhichao Liu,
National Center for Toxicological Research (FDA), United States

***Correspondence:**
Jiann Shiun Yuan
jiann-shiun.yuan@ucf.edu

Specialty section:
This article was submitted to Medicine and Public Health, a section of the journal Frontiers in Artificial Intelligence

Received: 09 May 2020
Accepted: 17 July 2020
Published: 18 August 2020

Citation:
Keshavarzi Arshadi A, Webb J, Salem M, Cruz E, Calad-Thomson S, Ghadirian N, Collins J, Diez-Cecilia E, Kelly B, Goodarzi H and Yuan JS (2020) Artificial Intelligence for COVID-19 Drug Discovery and Vaccine Development. Front. Artif. Intell. 3:65. doi: 10.3389/frai.2020.00065

SARS-COV-2 has roused the scientific community with a call to action to combat the growing pandemic. At the time of this writing, there are as yet no novel antiviral agents or approved vaccines available for deployment as a frontline defense. Understanding the pathobiology of COVID-19 could aid scientists in their discovery of potent antivirals by elucidating unexplored viral pathways. One method for accomplishing this is the leveraging of computational methods to discover new candidate drugs and vaccines *in silico*. In the last decade, machine learning-based models, trained on specific biomolecules, have offered inexpensive and rapid implementation methods for the discovery of effective viral therapies. Given a target biomolecule, these models are capable of predicting inhibitor candidates in a structural-based manner. If enough data are presented to a model, it can aid the search for a drug or vaccine candidate by identifying patterns within the data. In this review, we focus on the recent advances of COVID-19 drug and vaccine development using artificial intelligence and the potential of intelligent training for the discovery of COVID-19 therapeutics. To facilitate applications of deep learning for SARS-COV-2, we highlight multiple molecular targets of COVID-19, inhibition of which may increase patient survival. Moreover, we present CoronaDB-AI, a dataset of compounds, peptides, and epitopes discovered either *in silico* or *in vitro* that can be potentially used for training models in order to extract COVID-19 treatment. The information and datasets provided in this review can be used to train deep learning-based models and accelerate the discovery of effective viral therapies.

Keywords: COVID-19, SARS-COV-2, drug, vaccine, artificial intelligence, deep learning

INTRODUCTION

Coronaviridae is a viral family responsible for causing pneumonia-like symptoms that has been a global threat since its first outbreak in 2002 (Jabeer Khan et al., 2020). Severe Acute Respiratory Disease (SARS) and Middle Eastern Respiratory Syndrome (MERS), emerging in 2002 and 2013, respectively, caused diseases marked by both gastrointestinal and pulmonary dysfunction (Hilgenfeld and Peiris, 2013). In 2019, SARS-COV-2 was the causative agent of a third Coronavirus outbreak and has been identified as the virus responsible for COVID-19, the symptoms of which range from those of the common cold to more severe respiratory failure (Kong W.-H. et al., 2020). Despite its having been declared a pandemic by the World Health Organization (WHO), COVID-19 has continued to spread and has infected at least 20 million individuals, reaching a death toll of over half a million at the time of this review (Worldometer, 2020).

While hospitals are resorting to trial and error tactics for COVID-19 drug discovery, Virtual Screening (VS) has emerged as a popular method for discovering potent compounds due to the inefficiency of lab-based high throughput screening (HTS) (Jin et al., 2020; Kandeel and Al-Nazawi, 2020). VS for rational drug discovery is essentially an approach that involves computationally targeting a specific biomolecule (e.g., DNA, protein, RNA, lipid) of a cell to inhibit its growth and/or activation (Shoichet, 2004; Lionta et al., 2014). Structure-based and ligand-based drug discovery and design are two important subgroups of this type of screening (Lionta et al., 2014; Yu and Mackerell, 2017; Arshadi et al., 2020; Broom et al., 2020). Given our access to computationally and experimentally determined viral protein structures (Senior et al., 2020; Zhang L. et al., 2020), VS provides a rapid and cost-effective strategy for identifying antiviral candidates.

Additionally, conventional vaccine discovery methods have been costly, and it may take many years to develop an appropriate vaccine against a specified pathogen. In the early 1990s, the introduction of a genome-based vaccine design approach dubbed "Reverse Vaccinology" (RV) (Rappuoli, 2000; Bullock et al., 2020), revolutionized the field to a more efficient status, due in part to the fact that bacterial culturing was no longer required for identifying vaccine targets (Bruno et al., 2015; Heinson et al., 2015; Soria-Guerra et al., 2015). Moreover, all of the putative target protein antigens can be identified, rather than identification being limited to those isolated from bacterial cultures (Xiang and He, 2009; Bowman et al., 2011). All of these advantages taken together led scientists to generate RV prediction programs.

Over the past decade, artificial intelligence (AI)-based models have revolutionized drug discovery in general (Zhong et al., 2018; Duan et al., 2019; Lavecchia, 2019). AI has also led to the creation of many RV virtual frameworks, which are generally classified as rule-based filtering models (Naz et al., 2019; Ong et al., 2020a). Machine learning (ML) enables the creation of models that learn and generalize the patterns within the available data and can make inferences from previously unseen data. With the advent of deep learning (DL), the learning procedure can also include automatic feature extraction from raw data (Lecun et al., 2015). Moreover, it has recently been found that deep learning's feature extraction can result in superior performance compared to other computer-aided models (Ma et al., 2015; Chen et al., 2018; Zhavoronkov et al., 2019).

In this review, we provide a survey of AI-based models for COVID-19 drug discovery and vaccine development. Moreover, we identify and evaluate the best candidate targets for future treatment development. We propose that a concerted effort should be made to leverage the knowledge from pre-existing data by using machine learning approaches. To that end, we present a wide-ranging collection of small molecules, peptides, and epitopes for therapy discovery that could also direct AI-based models, screening, or generation, in an intelligent manner.

BACKGROUND OF MACHINE LEARNING METHODS FOR THERAPY DISCOVERY

In recent years, machine learning has revolutionized many fields of science and engineering. It has largely transformed our daily lives, from speech and face recognition (Alaghband et al., 2020; Grover and Toghi, 2020; Sun et al., 2020) to customized targeted advertisements (Zhai et al., 2016). The power of automatic abstract feature learning, combined with a massive volume of data, has immensely contributed to the successful application of ML (Lecun et al., 2015). Two of the most impactful areas affected are drug and vaccine discovery (Chen et al., 2018), in which ML has offered compound property prediction (Ma et al., 2015), activity prediction (Zhavoronkov et al., 2019), reaction prediction (Fooshee et al., 2018), and ligand–protein interaction.

On the prediction front, Graph Convolutional Neural Networks (GCNN) have been the favorite tool for drug discovery applications (Duvenaud et al., 2015; Kearnes et al., 2016). These networks are able to handle graphs and extract features via encoding the adjacency information within the features. Successful representation learning from molecules using GCNNs has been demonstrated in drug property prediction (Heskett et al., 2018; Bazgir et al., 2019; Liu et al., 2019), protein interface estimation (Fout et al., 2017), reactivity prediction (Coley et al., 2019), and drug–target interactions (Torng and Altman, 2019; Wang et al., 2020). Sequence-based models such as genomics, proteomics, and transcriptomics have also gained some attention in recent years due to the advancements made in the natural language processing domain. The more recent generation of context-based models are transformers that use attention mechanisms and self-supervision to extract representations from sequences (Vaswani et al., 2017; Devlin et al., 2018). Transformers have demonstrated the capacity to predict drug–target interactions (Shin et al., 2019), model protein sequences (Choromanski et al., 2020), and predict retrosynthetic reactions. These models learn to extract features from sequences on the location, context, and order of the input tokens (Belinkov and Glass, 2018). Recurrent neural networks (RNNs) and long short-term memory (LSTM) networks have successfully demonstrated the ability to perform when trained on molecules or protein sequences to predict secondary structure (Pollastri

et al., 2002), quantitative structure–activity relationship (QSAR) modeling (Chakravarti et al., 2019), and function prediction (Liu, 2017).

On the lead generation front, *de novo* design has benefitted the most from the application of deep learning. This subfield has drastically evolved from its traditional usage of ligand-based models and creating molecules from sub-blocks (Acharya et al., 2010). The current approach involves the use of state-of-the-art deep learning models such as Generative Adversarial Networks (GANs) to create data-oriented molecules (Guimaraes et al., 2017). Traditional *de novo* design fails to fully implement this exploration by constraining the generation of molecules with ligand or fragment libraries. More recent approaches utilize deep learning generative models such as variational autoencoders (VAE) (De Cao and Kipf, 2018) in order to create sequences of atoms. This approach lifts the constraints of ligand-based designs and allows the generation of unique molecules with greater diversity (Guimaraes et al., 2017; De Cao and Kipf, 2018; Jin et al., 2018; Liu et al., 2018; Simonovsky and Komodakis, 2018).

Machine learning has also improved the field of vaccine design over the past two decades. VaxiJen was the first implementation of ML in RV approaches and has shown promising results for antigen prediction (Doytchinova and Flower, 2007; Heinson et al., 2017). In addition, the recent development of Vaxign-ML, a web-based RV program leveraging machine learning approaches for bacterial antigen prediction, is a testament to the success of exercising mathematical ML-based in RV (He et al., 2010a; Heinson et al., 2017). In essence, these pipelines consist of feature extraction, feature selection, data augmentation, and cross-validation implemented to predict vaccine candidates against various bacterial and viral pathogens known to cause infectious disease. The use of biological, structural, and physiochemical features is prevalent among the approaches in this domain, as seen in reverse vaccinology and immunoinformatic methods such as IEDB and BlastP, which are feature extractors for AI-based models like RNN in the study of different pathogenic viruses (Flower et al., 2010; He and Zhu, 2015; Abbasi, 2020). More recently, graph-based features have also shown the ability to represent the antibodies instead of an expert-designed feature; Magar et al. showed that graph featurization is followed by mean pooling, and then classification is implemented using shallow and deep models (Magar et al., 2020). Deep Learning approaches have also revolutionized the field of cancer vaccinology through the improved prediction of neoantigens and their HLA binding affinity (Sher et al., 2017; Tran et al., 2019; Wu et al., 2019). Autoencoders of deep learning have shown promising improvement in extracting characteristics of human Leukocyte Antigen (HLA-A), which could be utilized in both transplantations and vaccine discovery (Miyake et al., 2018).

Key aspects of therapy discovery are safety and reliability. The Vaccine Adverse Event Reporting System (VAERS) and Vaccine Safety Databank (VSD) have been among the most popular immunization registries for tracking, recording, and predicting vaccine safety. In prior decades, implementations of computational simulation and mathematical modeling have significantly improved the tradeoff between the assessment of safety and efficacy by using the aforementioned resources (He et al., 2010b; Vaishnav et al., 2015). Zheng et al. implemented Natural language Processing (NLP) for the identification of adverse events related to Tdap vaccines (Zheng et al., 2019).

In drug development cases, the final drug candidate produced in the process of drug discovery needs to be safe for human consumption. This requires an observation of the drug's side effects as well as confirmation that the drug is non-toxic. To accomplish this, the Toxicology in the 21st Century program (Tox-21) has screened ∼10,000 compounds from 70 screening assays, creating a database that can be used to facilitate toxicity modeling. Furthermore, the project has also expanded to contain 700 assays with nearly 1,800 molecules in the ToxCast dataset. On the side-effect prevention front, the off-target interactions are predicted and minimized *in silico*. In doing so, potential drug candidates are chosen, with consideration given to their off-target polypharmacological profiles (Zhou H. et al., 2015). In a different approach, AI-based studies were implemented to detect the potential prolongation of QT intervals and cardiotoxicity of a candidate drug, hydroxychloroquine, using ECG data from smartwatches (Li J. et al., 2020)[1].

In summary, artificial intelligence has been applied to many subfields of drug discovery and vaccine development. This improvement is crucial for the current situation and immediate SARS-COV-2 therapy discovery for several key reasons. Firstly, the automatic feature extraction ability of deep learning can support models with better accuracy and deliver more reliable results. Secondly, the generative ability demonstrated by deep learning models can be utilized to create more druggable molecules and better epitope prediction, lowering the chance of failure in the trial pipeline. Lastly, the novelty of the virus causes the data around its possible therapies to be scarce, which is a suitable scenario for transfer learning and leveraging the learned knowledge from previous tasks (e.g., TranscreenTM) (Salem et al., 2020). Transfer learning has been shown to alleviate this problem through the transferring of learned knowledge and parameters from a secondary task with big data available to the task at hand (Weiss et al., 2016). Therefore, the use of deep learning in therapy discovery for SARS-COV-2 is essential in order to make a timely and accurate response to the virus.

COVID-19 MOLECULAR MECHANISM AND TARGET SELECTION

Coronaviruses are enveloped viruses with a positive-sense single-stranded RNA genome (Fehr and Perlman, 2015). They are known to infect both humans and other eukaryotes (Andersen et al., 2020; Hoffmann et al., 2020). The novel coronavirus manages to bind to the host receptor with a higher affinity than SARS due to the increased modification of its viral spike, among other structural proteins, resulting in enhanced transmission (Zhou Y. et al., 2020).

[1]AI study launched to monitor cardiac safety of COVID-19 patients receiving hydroxychloroquine. Available online at: https://cardiacrhythmnews.com/ai-study-launched-to-monitor-cardiac-safety-of-covid-19-patients-receiving-hydroxychloroquine/ (accessed July 04, 2020).

SARS-CoV-2 interaction with host cells begins with attachment via the viral spike (S) protein to the host ACE2 receptor (Hoffmann et al., 2020; Zhou P. et al., 2020). ACE2 binding induces the host surface serine protease, TMPRSS2, to prime the S protein via cleavage at its S1/S2 border, facilitating viral fusion with the cell membrane (Hoffmann et al., 2020). Once inside the cell, the viral RNA genome is released into the cytosol, where it is translated by host ribosome machinery, producing two polyproteins: pp1a and pp1ab, which are then cleaved by viral 3CL protease (main protease) and PL protease. This gives rise to several non-structural proteins (nsps) as the foundation of RNA-dependent RNA polymerase (RdRP); this RdRP then transcribes a template strand of the genomic RNA, from which it then transcribes subgenomic mRNA products to be translated. These products encode the structural proteins S, E, M, and N, as well as additional accessory nsps (**Figure 1**) (Lai and Cavanagh, 1997; Kim D. et al., 2020).

The severity of the host response depends on an innate response to viral recognition, involving the expression of type-1 IFNs and pro-inflammatory cytokines (Pazhouhandeh et al., 2018; Prompetchara et al., 2020). If the antiviral response is delayed or inhibited, viral proliferation can lead to the large-scale recruitment of neutrophils and monocyte-macrophages to the lungs, creating a hyperinflammatory environment (Prompetchara et al., 2020). Overactive release of pro-inflammatory cytokines, i.e., cytokine storm (CS), has been found in COVID-19 patients and can lead to severe complications like acute respiratory distress syndrome (ARDS) (Moore and June, 2020). It has been found that levels of IL-1B, IL-1RA, IL-8, IL-10, IFNγ, IP10, MCP1, and MIP1s are higher in COVID-19 patients than in healthy adults (Huang et al., 2020). IL-6, in particular, has been highly implicated in CRS and COVID-19 severity, and inhibition of IL-6/IL-6R activity may lead to improved patient outcome, increasing its desirability as a target (**Figure 1**) (Scheller et al., 2014; Tanaka et al., 2016; Zhang C. et al., 2020).

Throughout the process of viral entry, replication, and dissemination, there are several proteins that can serve as suitable targets for therapeutic intervention. The S protein is one of the candidates receiving the most focus, as it is necessary for viral entry into host cells and is highly specific to the virus itself. The host receptor ACE2 is another possible target, but the presence of ACE2 in non-lung tissues such as heart, kidney, and intestine (Hamming et al., 2004) could complicate its inhibition. Another host protein, the TMPRSS2 protease, is essential for viral entry into the cell, making it an additional viable target (Hoffmann et al., 2020).

COVID-19 DRUG DISCOVERY

Protein-Based

The recent applications of Artificial Intelligence for COVID-19 include the virtual screening of both repurposed drug candidates and new chemical entities. For repurposed drugs, the goal has been to rapidly predict and exploit interconnected biological pathways or the off-target biology of existing medicines that are proven safe and can thus be readily tested in new clinical trials. In one of the early attempts, Gordon et al. paved the way for the repurposing of candidate drugs by experimentally identifying 66 human proteins linked with 26 SARS-CoV-2 proteins (Gordon et al., 2020). In addition to wet-lab approaches, network-based model simulation has been the main computational approach for analyzing the virus–host interactome (Messina et al., 2020). Li et al. identified 30 drugs for repurposing by analyzing the genome sequence of three main viral family members of the coronavirus and then relating them to the human disease-based pathways (Li X. et al., 2020). In a different approach, Zhou et al. offered a combination of network-based methodologies for repurposed drug combination (Zhou Y. et al., 2020).

UK-based BenevolentAI leveraged its AI-derived knowledge graph, which integrates biomedical data from structured and unstructured sources (Richardson et al., 2020). It targeted the inhibition of host protein AAK1 and identified Baricitinib, an approved drug for the treatment of rheumatoid arthritis (Stebbing et al., 2020). Similarly, Beck et al. published an application of their DL-based drug–target interaction model that predicted commercially available antiviral drugs that may target the SARS-COV-2-related protease and helicase (Beck et al., 2020a). Atomwise has also focused on targeting several SARS-CoV-2 protein binding sites that are highly conserved across multiple coronavirus species in an effort to develop new broad-spectrum antivirals. Using its *AtomNet*® deep convolutional neural network technology (Wallach et al., 2020), Atomwise is screening millions of virtual compounds against these diverse targets alongside 15 different partnerships with academic researchers that will test the predicted compounds in their *in vitro* assays[2].

There have been several other applications of multi-task deep learning models for identifying existing drugs that can target the main viral proteins, especially the main protease ($3CL^{pro}$) and spike protein (Hu et al., 2020; Kadioglu et al., 2020; Kim J. et al., 2020; Redka et al., 2020). One impressive example is Cyclica's creation and mining of PolypharmDB, a platform of known drugs and their predicted binding to human protein targets that uncovered off-target applications of 30 existing drugs against the viral protein $3CL^{pro}$ and the ACE2 binding site as two examples (Redka et al., 2020). At least two other applications of DL-based virtual screening for the SARS-CoV-2 main protease have been published and include the open sharing of newly predicted chemical structures (Bung et al., 2020; Zhang H. et al., 2020).

ML-aided molecular docking has been one of the most prevalent approaches for virtual screening. This process normally requires the following: (1) Dataset of Druglike or Approved Molecules, (2) Crystal Structure or Homology Model of the target, (3) Molecular Docking Program, and (4) Compute Resources (Ewing et al., 2001; Pagadala et al., 2017). Through docking, many molecules have been reported to fit the binding site of various SARS-CoV-2 proteins essential for viral replication

[2]Atomwise Partners with Global Research Teams to Pursue Broad-Spectrum Treatments Against COVID-19 and Future Coronavirus Outbreaks | Business Wire. Available online at: https://www.businesswire.com/news/home/20200521005238/en/Atomwise-Partners-Global-Research-Teams-Pursue-Broad-Spectrum (accessed June 28, 2020).

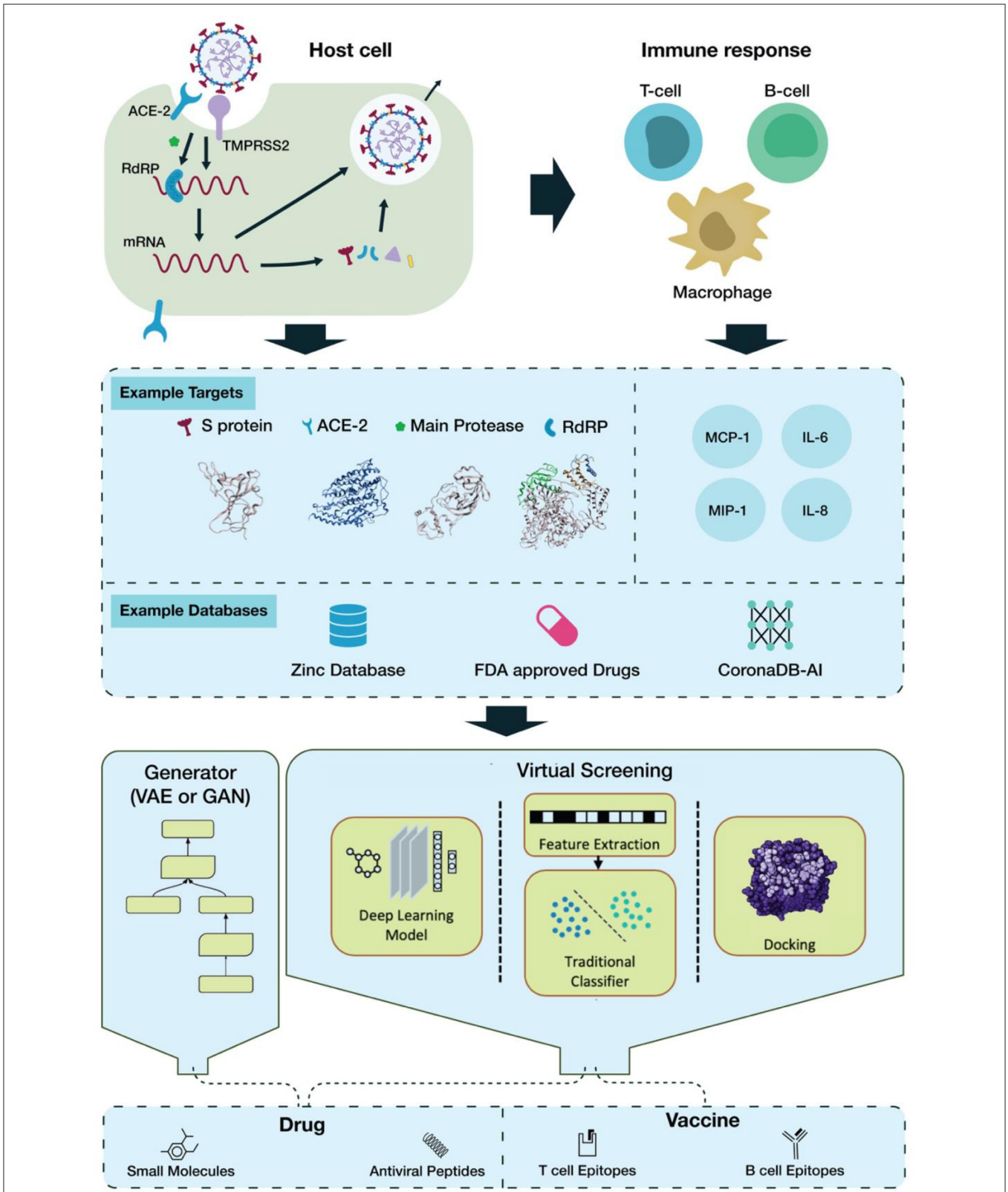


FIGURE 1 | The pipeline of AI-based drug discovery and vaccine development for COVID-19.

and infection. $3CL^{pro}$, Spike Protein, RdRP, and PL^{pro} are among those screened, as well as the host ACE2 receptor and TMPRSS2 protease (Chen et al., 2020; Choudhary et al., 2020; Kong R. et al., 2020; Smith and Smith, 2020; Wu et al., 2020). As an example, Ton et al. identified at least 1000 protease inhibitors by creating and utilizing the Deep Docking (DD) network technology approach. However, as they used the QSAR for training their model, no novel docking score was provided (Ton et al., 2020).

It is clear that $3CL^{pro}$ is the most popular target for virtual screening (**Figure 1**). The main reason for this is its pivotal role in viral replication and transcription and its well-defined structural information. Viral protease inhibitors have been extensively studied as treatments for other viruses. In addition, deep learning-aided approaches have been the main focus of research, as their automatic feature extraction accelerates discovery. The datasets cited often rely on the ZINC database (Wu et al., 2020), while other screened datasets include the FDA-approved LOPAC library (Choudhary et al., 2020), SWEETLEAD library (Smith and Smith, 2020), or all purchasable drugs (Drugs-lib) (Chen et al., 2020). Moreover this review sampled a variety of publications witch used different computational resources. It can be carried out on a small scale on a MacOS Mojave Workstation with an 8 core Zeon E5 processor or on a large scale as with the world's strongest supercomputer, SUMMIT, for enhanced parallelization (Choudhary et al., 2020; Smith and Smith, 2020).

RNA-Based

Conserved structured elements have already been shown to play critical functional roles in the life cycles of Coronaviruses (Yang and Leibowitz, 2015). Through direct interactions with host RNA-binding proteins and helicases, structural elements add a layer of complexity to the regulatory information that is encoded in the viral RNA. Targeted disruption of the regulatory functions of these structural elements provides a largely unexplored strategy that can limit viral loads with minimal impact on the biology of normal cells (Park et al., 2011). While this idea would have been farfetched a mere 5 years ago, advances in AI-driven computational modeling and high-throughput experimental RNA shape analyses have all but overcome the critical barriers (Alipanahi et al., 2015).

Highly conserved RNA structural elements have been identified in a number of viral families, many of which have been functionally validated (Jaafar and Kieft, 2019). Some of these stem loops in SARS-CoV-2′s 5′UTRs structural elements are conserved across beta coronaviruses and are known to impact viral replication (Yang and Leibowitz, 2015). There are many functional RNA structural elements that fall within the coding sequence and the 3′UTR as well (Plant and Dinman, 2008; Stammler et al., 2011). Rangan et al. identified 106 structurally conserved regions that would be suitable biotargets for unexplored antiviral agents (Rangan et al., 2020). Moreover, they predicted at least 59 unstructured regions that are conserved within SARS-CoV-2. Park et al. identified an RNA Pseudoknot-Binding molecule against SARS-CoV-1 in target-based virtual screening (Park et al., 2011; Nakagawa et al., 2016).

Studying the changes in RNA information also allows for the identification of new and evolved targets. In a different approach, Wu et al. showed that a recently FDA-approved drug named Remdesivir could bind to the RNA-binding channel of the novel coronavirus. They discovered other candidate drugs via analyzing the proteins critical to RNA processing and pathways (Wu et al., 2020). It seems that viral genome, RdRP, and processed mRNA would make promising targets for drug repurposing.

Generative Approaches

Molecule generation has been one of the fields of drug discovery that have been most revolutionized by the implementation of artificial intelligence over the last decade. As mentioned, VAE is a generator model for enhancing the diversity of generated data. Autoencoders instruct molecules into a vector that captures properties such as bond order, element, and functional group (Bjerrum and Sattarov, 2018). Chenthamarakshan et al., together with IBM Research, demonstrated a VAE that captures molecules in a latent space. Once captured, variations are made on the original molecule vectors based on desired properties. These can then be decoded back into novel molecules (Chenthamarakshan et al., 2020). To optimize the structures, QED, Synthetic Accessibility, and LogP regressors were used to improve the latent space variations.

In a different approach, Tang et al. overcame many of the issues with traditional generative models by developing a novel advanced deep Q-learning network with fragment-based drug design (ADQN-FBDD). This allowed for the enhanced exploration of space by assembling SARS-CoV-2 molecules one fragment at a time rather than relying on latent space adjustments. After making connections and rewarding molecules with the most druglike connections, a pharmacophore and descriptor filter was used to refine the set. They demonstrated a robust method for designing novel, high-binding compounds refined to the structure of SARS-CoV-2 $3CL^{Pro}$ (Tang et al., 2020). To design a drug-generative network, the following is necessary: (1) collection of Druglike Molecules, (2) a representation of these molecules *in silico* (i.e., Fingerprints, Tokenizers), (3) a method of altering molecules to increase diversity, and (4) screening and modification of the altered molecules. Pursuing GAN-related models, Insilico Medicine used three of its previously validated generative chemistry approaches to target the main protease, namely, crystal-derived pocked-based generation, homology modeling-based generation, and ligand-based generation (Zhavoronkov et al., 2020). Similar to target-based virtual screening, the main protease has been the main object of interest for scientists for *de novo* drug discovery.

COVID-19 VACCINE DISCOVERY

Identification of the best possible targets for the development of a vaccine is crucial in order to counteract a virus's high infection rate (Choudhary et al., 2020). A host immune system fights virus-infected cells either through the production of antibodies by B cells or through the direct attack of T cells (Amanat and Krammer, 2020). The HLA gene encodes MCH-I and MCH-II

proteins, which present epitopes as antigenic determinants. These proteins assist B-cell and T-cell antibodies in their ability to bind and attack invaders (Dangi et al., 2018; Gupta et al., 2020; Smith and Smith, 2020). Machine learning approaches, including Random Forest (RF), Support Vector Machine (SVM), and Recursive Feature Selection (RFE), have been basic tools for identifying antigens from protein sequences (Bowick et al., 2010; Rahman et al., 2019). However, due to their low sensitivity in the prediction of locally clustered interactions in some cases, Deep Convolutional Neural Networks (DCNN) have been a more valid alternative for the binding prediction of MHC and peptides (Han and Kim, 2017).

Since the outbreak of this first coronavirus, different AI-based approaches have been used to predict potential epitopes so as to design vaccines (Park et al., 2011; Yang and Leibowitz, 2015; Ton et al., 2020). Fast and Chen used MARIA (Chen et al., 2019) and NetMHCPan4 (Jurtz et al., 2017), two supervised neural network-driven tools, to discover potential T-cell epitopes for SARS-CoV-2 close to the 2019-nCoV spike receptor-binding domain (RBD) (Fast and Chen, 2020). The Long Short-Term Memory (LSTM) network has also shown some promising results. Abbasi et al. used this type of RNN to predict epitopes for Spike (Abbasi, 2020). Using a similar tactic, Crossman et al. employed deep-learning RNN and provided simulated sequences of Spike to identify possible targets for vaccine design (Crossman, 2020). RNN provided the sequences for a protein of interest with high sequence identity to the BLAST match.

Using a separate method, Feng et al. leveraged the iNeo tool to design a vaccine containing both B-cell and T-cell epitopes. This multi-peptide vaccine could provide a new strategy against SARS-CoV-2. Additionally, they discovered 17 vaccine peptides involving both immune cells (Nakagawa et al., 2016; Rangan et al., 2020). Ong et al. used Vaxign-RV to prioritize non-structural proteins as vaccine candidates for SARS-CoV-2 (Ong et al., 2020b). Nsp3, the largest non-structural protein of the coronavirus family, was identified as the most promising potential target for vaccine development after Spike (Ong et al., 2020b). Malone et al. also studied the entire SARS-CoV-2 proteome beyond Spike and provided a comprehensive vaccine design blueprint for SARS-CoV-2 using *NEC Immune* Profiler, IEDB, and BepiPred tools to create an epitope map for different HLA alleles (Malone et al., 2020).

Natural language processing models, specifically language modeling techniques, have also made an impact in the domain of COVID-19 vaccine discovery. Pre-trained transformers were used to predict protein interaction (Nambiar et al., 2020) and model molecular reactions in carbohydrate chemistry (Pesciullesi et al., 2020), which can be utilized in the process of vaccine development. Chen et al. discussed the use-case of an LSTM-based seq-2-seq model for predicting the secondary structure of certain SARS-COV-2 proteins (Karpov et al., 2019)[3]. Also, Beck et al. used transformers to repurpose commercially available drugs by predicting their interactions with viral proteins of SARS-COV-2 (Beck et al., 2020b).

[3]OSF Preprints. ZeroFold-Understanding Mutations of SARS-CoV-2 Spike Protein base on Secondary Structure Event Extracting for guiding Vaccine development. Available online at: https://osf.io/3vkuw/ (accessed Jul. 01, 2020).

Taking this work together, it is clear that spike protein has been the most popular candidate for virtual vaccine discovery (Oany et al., 2014). As the spike protein of SARS-COV-2 is crucial for viral entry, specific neutralizing antibodies against the receptor-binding domain of Spike can interrupt the attachment and fusion of viral proteins (Wan et al., 2019). This method could provide simulated sequences that can serve as a guide for further vaccine discovery against COVID-19 and possibly new zoonosis that may arise in the future.

DATA COLLECTION

Data-driven solutions rely on patterns embedded in the data in order to extract mathematical models. That being said, a data collection campaign will face a plethora of challenges in the case of any recently emerged virus, primarily due to the existence of bias and imbalance in the limited data available. Therefore, even the most sophisticated of modeling approaches will be ineffective when trained on such datasets. In order to overcome this issue, we compiled a multifaceted and comprehensive investigation of the existing literature, datasets, and online resources to provide potential small molecules, peptides, and epitopes. Such elements can be beneficial in the process of discovering or designing novel drugs to treat COVID-19 when used with both conventional and data-driven AI-based approaches.

We choose to focus on both potential antiviral agents and host biotarget inhibitors. The provided data entitled CoronaDB-AI in **Table 1** includes the small molecules and peptides proposed by both *in-silico* and *in-vitro* approaches. In addition to candidate scaffolds against the coronavirus's structural proteins, the potential inhibition of other respiratory tract viruses is taken into consideration to increase the therapeutic potential. Antimicrobial peptides have been validated as potent antivirals that disrupt either the viral membrane or an additional molecular mechanism of the virus (Akaji et al., 2011; Han and Kraí, 2020; Xia et al., 2020). As described before, the cytokine storm and an elevated immune response of the host plays a vital role in disease complication, so candidate immunosuppressants were also added as host-targeted agents. In addition to the potency of a candidate drug, it is crucial that the drug have high selectivity and low toxicity. Therefore, we also gathered a complete toxicity dataset from distinct databases, including ToxCast and Tox21. Finally, we gathered a comprehensive epitope-based dataset that could also guide deep learning-based models for improved vaccine development and epitope generation.

DISCUSSION

SARS-COV-2 rapidly transformed into a global challenge, costing thousands of lives, overwhelming healthcare systems, and threatening the economy all around the world. As we demonstrated above, it can be extremely challenging to experimentally perform a comprehensive potency evaluation of all drug and vaccine candidates in a timely fashion. We believe that leveraging computational models capable of filtering and generating reliable therapies can significantly speed up these discovery efforts. Employing artificial neural networks

TABLE 1 | CoronaDB-AI is a collection of small molecules, peptides, and epitopes for the purpose of COVID-19 therapy discovery.

Data provided	Discovery	Type	Mechanism of action	References
ANTIVIRAL DATA				
Total of 59,107		**Small molecules and peptides**		
50,000	*In-silico*	Small molecule	Antiviral	1
3,000	*In-silico*	Small molecule	Anti SARS2 protein	Chenthamarakshan et al., 2020
1,000	*In-silico*	Small molecule	Anti-protease	Ton et al., 2020
406	*In-vitro*	Small molecule	Inhibiting autophagy	2
802	*In-vitro*	Small molecule	Activating autophagy	2
393	*In-vitro*	Small molecule	Biotargets of coronaviruses	3
110	*In-vitro*	Peptide and small molecule	Coronavirus and respiratory disease	Pillaiyar et al., 2020
1,000	*In-silico*	Small molecule	3C protease inhibitor	Zhavoronkov et al., 2020
11	*In-silico*	Small molecule	Main protease inhibitor	Fischer et al., 2020
20	*In-vitro*	Antimicrobial peptide	Anti-SARS/MERS	Mustafa et al., 2018
7	*In-silico*	Antimicrobial peptide	Anti-MERS	Mustafa et al., 2019
277	*In-vitro*	Antimicrobial peptide	Antiviral	Wang et al., 2015
4	*In-silico*	Antimicrobial peptide	Anti-spike of sars-Cov-2	Han and Kraí, 2020
379	*In-vitro*	Small molecule	Anti-respiratory syncytial virus	Plant et al., 2015
13	*In-vitro*	Small molecule	Anti-recurrent respiratory papillomatosis by HPV-6	Alkhilaiwi et al., 2019
1,280	*In-vitro*	Small molecule	Anti-respiratory syncytial virus	Rasmussen et al., 2011
16	*In-silico*	Small molecules	Anti-SARS-COV-2	Zhou Y. et al., 2020
77	*In-silico*	Small molecules	Anti-S Protein of SARS-COV-2	Smith and Smith, 2020
10	*In-silico*	Small molecules	Anti-SARS-COV2	Hu et al., 2020
25	*In-silico*	Small molecules	Anti SARS2 Proteins	Kim J. et al., 2020
10	*In-silico*	Small molecules	ACE2 and Spike inhibitors	Choudhary et al., 2020
78	*In-silico*	Small molecules	All SARS2 proteins	Wu et al., 2020
47	*In-silico*	Small molecules	3cl protease and M pro	Tang et al., 2020
16	*In-silico*	Small molecules	3cl protease inhibitor	Chen et al., 2020
36	*In-vitro*	Small molecules	Anti- Coronavirus-OC43	Shen et al., 2019
90	*In-vitro*	Small molecules	Anti- SARS-COV-2	Touret et al., 2020
ANTI-HOST PROTEINS				
Total of 677		**Small molecules and peptides**		
6	*In-vitro*	Small molecules	Anti-IL-1β and TNFα	Laufer et al., 2002
182	*In-vitro*	Peptides	Cytokine Signaling Inhibitors	4
269	*In-silico*	Small molecules	Anti-IL-6	Shukla et al., 2019
121	*In-vitro*	Small molecules	Severe acute respiratory	5
69	*In-silico*	Small molecules	Anti-protein-protein interaction of virus-host	Gordon et al., 2020
30	*In-silico*	Small molecules	Anti-host & virus interaction	Redka et al., 2020
TOXICITY DATA				
Total of 25,333		**Small molecules**		
11,800	*In-vitro*	Small molecules	Tox21 and ToxCast	Toxicology, EPA's National Center for Computational, 2018
13,533	*In-vitro*	Small molecules	Toxic for HepG2 Cell Line	Gamo et al., 2010
VACCINE DATA				
Total of 517		**Epitopes and vaccines**		
162	*In-silico*	Epitopes	Anti-SARS-COV-2	Ahmed et al., 2020
174	*In-silico*	Epitope	Anti-SARS-COV-2	Prachar et al., 2020
2	*In-silico*	Epitope	Anti-SARS-COV-2	Fast and Chen, 2020
30	*In-silico*	Vaccine candidate	Anti-SARS-COV-2	Feng et al., 2020
7	*In-silico*	Epitope	Anti-SARS-COV-2	Lon et al., 2020
12	*In-silico*	Epitope	Anti-SARS-COV-2	Tilocca et al., 2020
59	*In-silico*	Epitope	Anti-SARS-COV-2	Sarkar et al., 2020
71	*In-silico*	Epitope	Anti-SARS-COV-2	Bhattacharya et al., 2020

[1]*Download CAS COVID-19 Antiviral Candidate Compounds Dataset | CAS. Available online at: https://www.cas.org/covid-19-antiviral-compounds-dataset (accessed April 27, 2020).*
[2]*Novel Coronavirus Information Center. Available online at: https://www.elsevier.com/connect/coronavirus-information-center (accessed April 27, 2020).*
[3]*https://www.elsevier.com/__data/assets/pdf_file/0004/978745/Copy-of-RMC-substances-coronovirus-targets-pX6.pdf (accessed April 27, 2020).*
[4]*Cytokines Inhibitor library|Targetmol|96-well. Available online at: https://www.targetmol.com/compound-library/Cytokines-inhibitors-Library (accessed April 27, 2020).*
[5]*https://www.elsevier.com/__data/assets/pdf_file/0007/977173/ResNet-Data_Coronavirus.pdf (accessed April 27, 2020).*

and supervised learning methods has proven to be a vital game-changer when used for the purpose of virtual filtering and *de novo* design. However, in order to achieve the desired performance in such intelligent methods, one requires the knowledge to recognize the most relevant biotargets in addition to a large-scale training dataset. This fact motivated us to perform a survey of biotargets that have been employed in the virtual drug and vaccine discovery literature. We observed that the viral spike protein and the main protease have been the most prevalent choices for vaccine development and drug discovery, respectively, due to their importance. Furthermore, we gathered a list of datasets titled "CoronaDB-AI" that can be used for our particular application. Having access to these key elements removes the burden of collecting training data and the required knowledge for both computer scientists and bioinformaticians and consequently enhances research outcomes.

AUTHOR CONTRIBUTIONS

AK organized and wrote most of article and gathered all the data. JW contributed to the molecular part. MS contributed to the background for AI-based methods. EC, ED-C, and BK from A2A and SC-T from Atomwise contributed to the COVID19 drug discovery. NG and JC contributed to the vaccine discovery. HG contributed to the RNA-based and molecular sections. JY provided guidance in the opportunities of deep learning in a multidiscipline collaboration. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

We thank Farnam Kavehei for designing the figure. Also, we thank Melana Francisco for her contribution to the introduction of the article.

REFERENCES

Abbasi, B. A. (2020). Identification_of_vaccine_targets_and_design_of_vaccine_against_SARS. *OSF Preprints*. doi: 10.31219/osf.io/f8zyw

Acharya, C., Coop, A., Polli, J. E., and MacKerell, A. D. (2010). Recent advances in ligand-based drug design: relevance and utility of the conformationally sampled pharmacophore approach. *Curr. Comput. Aided-Drug Des.* 7, 10–22. doi: 10.2174/157340911793743547

Ahmed, S. F., Quadeer, A. A., and McKay, M. R. (2020). Preliminary identification of potential vaccine targets for the COVID-19 Coronavirus (SARS-CoV-2) Based on SARS-CoV immunological studies. *Viruses* 12:253. doi: 10.3390/v12030254

Akaji, K., Konno, H., Mitsui, H., Teruya, K., Shimamoto, Y., Hattori, Y., et al. (2011). Structure-based design, synthesis, and evaluation of peptide-mimetic SARS 3CL protease inhibitors. *J. Med. Chem.* 54, 7962–7973. doi: 10.1021/jm200870n

Alaghband, M., Yousefi, N., and Garibay, I. (2020). FePh: an annotated facial expression dataset for the RWTH-PHOENIX-weather 2014 Dataset. *arXiv: 2003.08759v1*. Available online at: https://arxiv.org/pdf/2003.08759.pdf

Alipanahi, B., Delong, A., Weirauch, M. T., and Frey, B. J. (2015). Predicting the sequence specificities of DNA- and RNA-binding proteins by deep learning. *Nat. Biotechnol.* 33, 831–838. doi: 10.1038/nbt.3300

Alkhilaiwi, F., Paul, S., Zhou, D., Zhang, X., Wang, F., Palechor-Ceron, N., et al. (2019). High-throughput screening identifies candidate drugs for the treatment of recurrent respiratory papillomatosis. *Papillomavirus Res.* 8:100181. doi: 10.1016/j.pvr.2019.100181

Amanat, F., and Krammer, F. (2020). SARS-CoV-2 vaccines: status report. *Immunity* 52, 583–589. doi: 10.1016/j.immuni.2020.03.007

Andersen, K. G., Rambaut, A., Lipkin, W. I., Holmes, E. C., and Garry, R. F. (2020). The proximal origin of SARS-CoV-2. *Nat. Med.* 26, 450–452. doi: 10.1038/s41591-020-0820-9

Arshadi, A. K., Salem, M., Collins, J., Yuan, J. S., and Chakrabarti, D. (2020). Deepmalaria: artificial intelligence driven discovery of potent antiplasmodials. *Front. Pharmacol.* 10:1526. doi: 10.3389/fphar.2019.01526

Bazgir, O., Zhang, R., Rahman Dhruba, S., Rahman, R., Ghosh, S., and Pal, R. (2019). REFINED (REpresentation of Features as Images With NEighborhood Dependencies): a novel feature representation for convolutional neural networks. *arXiv [Preprint] arXiv*:1912.05687 (2019).

Beck, B. R., Shin, B., Choi, Y., Park, S., and Kang, K. (2020a). Predicting commercially available antiviral drugs that may act on the novel coronavirus (2019-nCoV), Wuhan, China through a drug-target interaction deep learning model. *bioRxiv [Preprint]*. doi: 10.1101/2020.01.31.929547

Beck, B. R., Shin, B., Choi, Y., Park, S., and Kang, K. (2020b). Predicting commercially available antiviral drugs that may act on the novel coronavirus (SARS-CoV-2) through a drug-target interaction deep learning model. *Comput. Struct. Biotechnol. J.* 18, 784–790. doi: 10.1016/j.csbj.2020.03.025

Belinkov, Y., and Glass, J. (2018). Analysis methods in neural language processing: a survey. *Trans. Assoc. Comput. Linguist.* 7, 49–72. doi: 10.1162/tacl_a_00254

Bhattacharya, M., Sharma, A. R., Patra, P., Ghosh, P., Sharma, G., Patra, B. C., et al. (2020). Development of epitope-based peptide vaccine against novel coronavirus (2019). (SARS-COV-2): immunoinformatics approach. *J. Med. Virol.* 92, 618–631. doi: 10.1002/jmv.25736

Bjerrum, E. J., and Sattarov, B. (2018). Improving chemical autoencoder latent space and molecular de novo generation diversity with heteroencoders. *Biomolecules* 8:131. doi: 10.3390/biom8040131

Bowick, G. C., and Barrett, A. D., T. (2010). Comparative pathogenesis and systems biology for biodefense virus vaccine development. *J. Biomed. Biotechnol.* (2010) 2010:236528. doi: 10.1155/2010/236528

Bowman, B. N., McAdam, P. R., Vivona, S., Zhang, J. X., Luong, T., Belew, R. K., et al. (2011). Improving reverse vaccinology with a machine learning approach. *Vaccine* 29, 8156–8164. doi: 10.1016/j.vaccine.2011.07.142

Broom, A., Rakotoharisoa, R. V., Thompson, M. C., Zarifi, N., Nguyen, E., Mukhametzhanov, N., et al. (2020). Evolution of an enzyme conformational ensemble guides design of an efficient biocatalyst. *bioRxiv [Preprint]*. doi: 10.1101/2020.03.19.999235

Bruno, L., Cortese, M., Rappuoli, R., and Merola, M. (2015). Lessons from Reverse Vaccinology for viral vaccine design. *Curr. Opin. Virol.* 11, 89–97. doi: 10.1016/j.coviro.2015.03.001

Bullock, J., Alexandra, L., Pham, K. H., Lam, C. S. N., and Luengo-Oroz, M. (2020). Mapping the landscape of artificial intelligence applications against COVID-19. *arXiv [Preprint] arXiv:*2003.11336 (2020).

Bung, N., Krishnan, S. R., Bulusu, G., and Roy, A. (2020). *De Novo* design of new chemical entities (NCEs) for SARS-CoV-2 using artificial intelligence. *ChemRxiv [Preprint]*. doi: 10.26434/chemrxiv.11998347.v2

Chakravarti, S. K., and Alla, S. R., M. (2019). Descriptor free QSAR modeling using deep learning with long short-term memory neural networks. *Front. Artif. Intell.* 2:17. doi: 10.3389/frai.2019.00017

Chen, B., Khodadoust, M. S., Olsson, N., Wagar, L. E., Fast, E., Liu, C. L., et al. (2019). Predicting HLA class II antigen presentation through integrated deep learning. *Nat. Biotechnol.* 37, 1332–1343. doi: 10.1038/s41587-019-0280-2

Chen, H., Engkvist, O., Wang, Y., Olivecrona, M., and Blaschke, T. (2018). The rise of deep learning in drug discovery. *Drug Discov. Today* 23, 1241–1250. doi: 10.1016/j.drudis.2018.01.039

Chen, Y. W., Yiu, C.-P. B., and Wong, K.-Y. (2020). Prediction of the SARS-CoV-2 (2019-nCoV) 3C-like protease (3CLpro) structure: virtual screening reveals velpatasvir, ledipasvir, and other drug repurposing candidates. *F1000Research* 9:129. doi: 10.12688/f1000research.22457.2

Chenthamarakshan, V., Das, P., Padhi, I., Strobelt, H., Lim, K. W., Hoover, B., et al. (2020). *Target-Specific and Selective Drug Design for COVID-19 Using Deep Generative Models*. Available: http://arxiv.org/abs/2004.01215 (accessed April 19, 2020).

Choromanski, K., Likhosherstov, V., Dohan, D., Song, X., Davis, J., Sarlos, T., et al. (2020). *Masked Language Modeling for Proteins via Linearly Scalable Long-Context Transformers*. Available online at: http://arxiv.org/abs/2006.03555 (accessed July 01, 2020).

Choudhary, S., Malik, Y. S., and Tomar, S. (2020). Identification of SARS-CoV-2 cell entry inhibitors by drug repurposing using *in silico* structure-based virtual screening approach. *ChemRxiv [Preprint]*. doi: 10.3389/fimmu.2020.01664

Coley, C. W., Jin, W., Rogers, L., Jamison, T. F., Jaakkola, T. S., Green, W. H., et al. (2019). A graph-convolutional neural network model for the prediction of chemical reactivity. *Chem. Sci.* 10, 370–377. doi: 10.1039/C8SC04228D

Crossman, L. C. (2020). Leverging deep learning to simulate coronavirus spike proteins has the potential to predict future Zoonotic sequences. *bioRxiv [Preprint]*. doi: 10.1101/2020.04.20.046920

Dangi, M., Kumari, R., Singh, B., and Chhillar, A. K. (2018). Advanced *in silico* tools for designing of antigenic epitope as potential vaccine candidates against coronavirus. *Bioinforma. Seq. Struct. Phylogeny*. 329–357. doi: 10.1007/978-981-13-1562-6_15

De Cao, N., and Kipf, T. (2018). MolGAN: An implicit generative model for small molecular graphs. Available online at: http://arxiv.org/abs/1805.11973 (accessed April 26, 2020).

Devlin, J., Chang, W.-M., Lee, K., Google, K. T., and Language, A. I. (2018). BERT: pre-Training of deep bidirectional transformers for language understanding. *arXiv [preprint] arXiv*:1810.04805 (2018).

Doytchinova, I. A., and Flower, D. R. (2007). VaxiJen: a server for prediction of protective antigens, tumour antigens and subunit vaccines. *BMC Bioinformatics* 8:4. doi: 10.1186/1471-2105-8-4

Duan, Y., Edwards, J. S., and Dwivedi, Y. K. (2019). Artificial intelligence for decision making in the era of Big Data – evolution, challenges and research agenda. *Int. J. Inf. Manage.* 48, 63–71. doi: 10.1016/j.ijinfomgt.2019.01.021

Duvenaud, D., Maclaurin, D., Aguilera-Iparraguirre, J., Gómez-Bombarelli, R., Hirzel, T., Aspuru-Guzik, A., et al. (2015). Convolutional networks on graphs for learning molecular fingerprints. *arXiv:1509.09292*.

Ewing, T. J. A., Makino, S., Skillman, A. G., and Kuntz, I. D. (2001). DOCK 4.0: Search strategies for automated molecular docking of flexible molecule databases. *J. Comput. Aided. Mol. Des.* 15, 411–428. doi: 10.1023/A:1011115820450

Fast, E., and Chen, B. (2020). Potential T-cell and B-cell Epitopes of 2019-nCoV. *bioRxiv [Preprint]*. doi: 10.1101/2020.02.19.955484

Fehr, A. R., and Perlman, S. (2015). "Coronaviruses: an overview of their replication and pathogenesis," in *Coronaviruses: Methods and Protocols 1282*. New York, NY: Springer, 1–23. doi: 10.1007/978-1-4939-2438-7_1

Feng, Y., Qiu, M., Zou, S., Li, Y., Luo, K., Chen, R., et al. (2020). Multi-epitope vaccine design using an immunoinformatics approach for 2019 novel coronavirus in China (SARS-CoV-2). *bioRxiv [Preprint]*. doi: 10.1101/2020.03.03.962332

Fischer, A., Sellner, M., Neranjan, S., Lill, M. A., and Smieško, M. (2020). Inhibitors for novel coronavirus protease identified by virtual screening of 687 million compounds. *ChemRxiv [Preprint]*. doi: 10.26434/chemrxiv.11923239.v1

Flower, D. R., MacDonald, I. K., Ramakrishnan, K., Davies, M. N., and Doytchinova, I. A. (2010). Computer aided selection of candidate vaccine antigens. *Immunome Res.* 6(Suppl. 2), 1–16. doi: 10.1186/1745-7580-6-S2-S1

Fooshee, D., Mood, A., Gutman, E., Tavakoli, M., Urban, G., Liu, F., et al. (2018). Deep learning for chemical reaction prediction. *Mol. Syst. Des. Eng.* 3, 442–452. doi: 10.1039/C7ME00107J

Fout, A., Byrd, J., Shariat, B., and Ben-Hur, A. (2017). "Protein interface prediction using graph convolutional networks," in *Advances in Neural Information Processing Systems* (Long Beach, CA), 6530–6539.

Gamo, F.-J., Sanz, L. M., Vidal, J., de Cozar, C., Alvarez, E., Lavandera, J.-L., et al. (2010). Thousands of chemical starting points for antimalarial lead identification. *Nature* 465, 305–310. doi: 10.1038/nature09107

Gordon, D. E., Jang, G. M., Bouhaddou, M., Xu, J., Obernier, K., O'Meara, M. J., et al. (2020). A SARS-CoV-2-human protein-protein interaction map reveals drug targets and potential drug-repurposing. *bioRxiv [Preprint]*. doi: 10.1101/2020.03.22.002386

Grover, D., and Toghi, B. (2020). MNIST dataset classification utilizing k-NN classifier with modified sliding-window metric. *Adv. Intel. Syst. Comp.* 944, 583–591. doi: 10.1007/978-3-030-17798-0_47

Guimaraes, G. L., Sanchez-Lengeling, B., Outeiral, C., Farias, L. C., and Aspuru-Guzik, A. (2017). *Objective-Reinforced Generative Adversarial Networks (ORGAN) for Sequence Generation Models.* Available online at: http://arxiv.org/abs/1705.10843 (accessed April 26, 2020).

Gupta, E., Mishra, R. K., and Niraj, R. R. K. (2020). Identification of potential vaccine candidates against *SARS-CoV-2*, a step forward to fight novel coronavirus 2019-nCoV: a reverse vaccinology approach. *bioRxiv [Preprint]*. doi: 10.1101/2020.04.13.039198

Hamming, I., Timens, W., Bulthuis, M. L, C., Lely, A. T., Navis, G. J., and van Goor, H. (2004). Tissue distribution of ACE2 protein, the functional receptor for SARS coronavirus. A first step in understanding SARS pathogenesis. *J. Pathol.* 203, 631–637. doi: 10.1002/path.1570

Han, Y., and Kim, D. (2017). Deep convolutional neural networks for pan-specific peptide-MHC class I binding prediction. *BMC Bioinformat.* 18:585. doi: 10.1186/s12859-017-1997-x

Han, Y., and Kraí, P. (2020) Computational design of ACE2-based peptide inhibitors of SARS-CoV-2. *ACS Nano* 14, 5143–5147. doi: 10.1021/acsnano.0c02857

He, L., and Zhu, J. (2015). Computational tools for epitope vaccine design and evaluation. *Curr. Opin. Virol.* 11, 103–112. doi: 10.1016/j.coviro.2015.03.013

He, Y., Rappuoli, R., De Groot, A. S., and Chen, R. T. (2010b). Emerging vaccine informatics. *J. Biomed. Biotechnol.* doi: 10.1155/2010/218590

He, Y., Xiang, Z., and Mobley, H. L. T. (2010a). Vaxign: the first web-based vaccine design program for reverse vaccinology and applications for vaccine development. *J. Biomed. Biotechnol.* 2010:297505. doi: 10.1155/2010/297505

Heinson, A. I., Gunawardana, Y., Moesker, B., Denman Hume, C. C., Vataga, E., Hall, Y., et al. (2017). Enhancing the biological relevance of machine learning classifiers for reverse vaccinology. *Int. J. Mol. Sci.* 18:312. doi: 10.3390/ijms18020312

Heinson, A. I., Woelk, C. H., and Newell, M. L. (2015). The promise of reverse vaccinology. *Int. Health* 7, 85–89. doi: 10.1093/inthealth/ihv002

Heskett, C., Faircloth, B., Roper, S., and Clay, M. (2018). *Executive Insights Artificial Intelligence in Life Sciences: The Formula for Pharma Success Across the Drug Lifecycle*. Available online at: https://www.lek.com/sites/default/files/insights/pdf-attachments/2060-AI-in-Life-Sciences.pdf (accessed June 18, 2019).

Hilgenfeld, R., and Peiris, M. (2013). From SARS to MERS: 10 years of research on highly pathogenic human coronaviruses. *Antivir. Res.* 100, 286–295. doi: 10.1016/j.antiviral.2013.08.015

Hoffmann, M., Kleine-Weber, H., Schroeder, S., Kruger, N., Herrler, T., Erichsen, S., et al. (2020). SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor. *Cell* 181, 271-280.e8. doi: 10.1016/j.cell.2020.02.052

Hu, F., Jiang, J., and Yin, P. (2020). *Prediction of Potential Commercially Inhibitors Against SARS-CoV-2 by Multi-Task Deep Model.* Available online at: https://arxiv.org/ftp/arxiv/papers/2003/2003.00728.pdf (accessed April 22, 2020).

Huang, C., Wang, Y., Li, X., Ren, L., Zhao, J., Hu, Y., et al. (2020). Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet* 395, 497–506. doi: 10.1016/S0140-6736(20)30183-5

Jaafar, Z. A., and Kieft, J. S. (2019). Viral RNA structure-based strategies to manipulate translation. *Nat. Rev. Microbiol.* 17, 110–123. doi: 10.1038/s41579-018-0117-x

Jabeer Khan, R., Kumar Jha, R., Muluneh Amera, G., Jain, M., Singh, E., Pathak, A., et al. (2020). Targeting novel coronavirus 2019: a systematic drug repurposing approach to identify promising inhibitors against 3C-like proteinase and 2'-O-ribose methyltransferase: a systematic drug repurposing approach to identify promising inhibitors against 3C-like proteinase and 2'-O-ribose methyltransferase. *ChemRxiv [Preprint]*. doi: 10.26434/chemrxiv.11888730.v1

Jin, W., Barzilay, R., and Jaakkola, T. (2018). Junction tree variational autoencoder for molecular graph generation. *arXiv [Preprint]. arXiv:*1802.04364.

Jin, Z., Du, X., Xu, Y., Deng, Y., Liu, M., Zhao, Y., et al. (2020). Structure of Mpro from COVID-19 virus and discovery of its inhibitors. *bioRxiv [Preprint]*. doi: 10.1101/2020.02.26.964882

Jurtz, V., Paul, S., Andreatta, M., Marcatili, P., Peters, B., and Nielsen, M. (2017). NetMHCpan-4.0: improved peptide–mhc class i interaction predictions

integrating eluted ligand and peptide binding affinity data. *J. Immunol.* 199, 3360–3368. doi: 10.4049/jimmunol.1700893

Kadioglu, O., Saeed, M., Greten, H. J., and Efferth, Y. (2020). Identification of novel compounds against three targets of SARS CoV2 coronavirus by combined virtual screening and supervised machine learning. *Bull World Heal. Organ.* doi: 10.2471/BLT.20.255943

Kandeel, M., and Al-Nazawi, M. (2020). Virtual screening and repurposing of FDA approved drugs against COVID-19 main protease. *Life Sci.* 251:117627. doi: 10.1016/j.lfs.2020.117627

Karpov, P., Godin, G., and Tetko, I. V. (2019). "A transformer model for retrosynthesis," in *Artificial Neural Networks and Machine Learning – ICANN 2019: Workshop and Special Sessions. ICANN 2019. Lecture Notes in Computer Science*, Vol. 11731, eds I. Tetko, V. Kurková, P. Karpov, and Theis F (Cham: Springer). doi: 10.1007/978-3-030-30493-5_78

Kearnes, S., McCloskey, K., Berndl, M., Pande, V., and Riley, P. (2016). Molecular graph convolutions: moving beyond fingerprints. *J. Comput. Aided. Mol. Des.* 30, 595–608. doi: 10.1007/s10822-016-9938-8

Kim, D., Lee, J.-Y., Yang, J.-S., Kim, J. W., Kim, V. N., and Chang, H. (2020). The architecture of SARS-CoV-2 transcriptome. *Cell.* 181, 914–921. doi: 10.1016/j.cell.2020.04.011

Kim, J., Zhang, J., Cha, Y., Kolitz, S., Funt, J., Escalante Chong, R., et al. (2020). Advanced bioinformatics rapidly identifies existing therapeutics for patients with coronavirus disease–2019 (COVID-19). *ChemRxiv [Preprint]*. doi: 10.26434/chemrxiv.12037416

Kong, R., Yang, G., Xue, R., Liu, M., Wang, F., Hu, J., et al. (2020). *COVID-19 Docking Server: An Interactive Server for Docking Small Molecules, Peptides and Antibodies Against Potential Targets of* COVID-19. Available online at: https://arxiv.org/abs/2003.00163 (accessed April 29, 2020). doi: 10.1093/bioinformatics/btaa645

Kong, W.-H., Li, Y., Peng, M.-W., Kong, D.-G., Yang, X.-B., Wang, L., et al. (2020). SARS-CoV-2 detection in patients with influenza-like illness. *Nat. Microbiol.* 5, 675–678. doi: 10.1038/s41564-020-0713-1

Lai, M. M., and Cavanagh, D. (1997). The molecular biology of coronaviruses. *Adv. Virus Res.* 48, 1–100. doi: 10.1016/S0065-3527(08)60286-9

Laufer, S., Greim, C., and Bertsche, T. (2002). An in-vitro screening assay for the detection of inhibitors of proinflammatory cytokine synthesis: A useful tool for the development of new antiarthritic and disease modifying drugs. *Osteoarthr. Cartil.* 10, 961–967. doi: 10.1053/joca.2002.0851

Lavecchia, A. (2019). Deep learning in drug discovery: opportunities, challenges and future prospects. *Drug Discovery Today* 24, 2017–2032. doi: 10.1016/j.drudis.2019.07.006

Lecun, Y., Bengio, Y., and Hinton, G. (2015). Deep learning. *Nature* 521, 436–444. doi: 10.1038/nature14539

Li, J., Shao, J., Wang, C., and Li, W. (2020). The epidemiology and therapeutic options for the COVID-19. *Precis. Clin. Med.* 3, 71–84. doi: 10.1093/pcmedi/pbaa017

Li, X., Yu, J., Zhang, Z., Ren, J., Peluffo, A. E., Zhang, W., et al. (2020). Network bioinformatics analysis provides insight into drug repurposing for COVID-2019. *Preprints* 1–15. doi: 10.20944/preprints202003.0286.v1

Lionta, E., Spyrou, G., Vassilatis, D., and Cournia, Z. (2014). Structure-based virtual screening for drug discovery: principles, applications and recent advances. *Curr. Top. Med. Chem.* 14, 1923–1938. doi: 10.2174/1568026614666140929124445

Liu, K., Sun, X., Jia, L., Ma, J., Xing, H., Wu, J., et al. (2019). Chemi-net: A molecular graph convolutional network for accurate drug property prediction. *Int. J. Mol. Sci.* 20:3389. doi: 10.3390/ijms20143389

Liu, Q., Allamanis, M., Brockschmidt, M., and Gaunt, A. L. (2018). "Constrained graph variational autoencoders for molecule design," in *Advances in Neural Information Processing Systems* (Montreal, QC), 7795–7804.

Liu, X. (2017). *Deep Recurrent Neural Network for Protein Function Prediction from* Sequence. Available online at: https://arxiv.org/abs/1701.08318 (accessed April 26, 2020). doi: 10.1101/103994

Lon, J. R., Bai, Y., Zhong, B., Cai, F., and Du, H. (2020). Prediction and evolution of B cell epitopes of surface protein in SARS-CoV-2. *bioRxiv [Preprint]*. doi: 10.1101/2020.04.03.022723

Ma, J., Sheridan, R. P., Liaw, A., Dahl, G. E., and Svetnik, V. (2015). Deep neural nets as a method for quantitative structure-activity relationships. *J. Chem. Inf. Model.* 55, 263–274. doi: 10.1021/ci500747n

Magar, R., Yadav, P., and Farimani, A. B. (2020). *Potential Neutralizing Antibodies Discovered for Novel Corona Virus Using Machine Learning.* Available onlin at: http://arxiv.org/abs/2003.08447 (accessed April 30, 2020). doi: 10.1101/2020.03.14.992156

Malone, B., Simovski, B., Moliné, C., Cheng, J., Fontenelle, H., Vardaxis, I., et al. (2020). Artificial intelligence predicts the immunogenic landscape of SARS-CoV-2: toward universal blueprints for vaccine designs. *bioRxiv [Preprint]*. doi: 10.1101/2020.04.21.052084

Messina, F., Giombini, E., Agrati, C., Vairo, F., Ascoli Bartoli, T., Al Moghazi, S., et al. (2020). COVID-19: viral-host interactome analyzed by network based-approach model to study pathogenesis of SARS-CoV-2 infection. *J. Transl. Med.* 18:233. doi: 10.1186/s12967-020-02405-w

Miyake, J., Kaneshita, Y., Asatani, S., Tagawa, S., Niioka, H., and Hirano, T. (2018). Graphical classification of DNA sequences of HLA alleles by deep learning. *Hum. Cell* 31, 102–105. doi: 10.1007/s13577-017-0194-6

Moore, B. J. B., and June, C. H. (2020). Cytokine release syndrome in severe COVID-19. *Science*. 368, 473–474. doi: 10.1126/science.abb8925

Mustafa, S., Balkhy, H., and Gabere, M. (2019). *Peptide-Protein Interaction Studies of Antimicrobial Peptides Targeting Middle East Respiratory Syndrome Coronavirus Spike Protein: An In Silico Approach*. London: Hindawi. doi: 10.1155/2019/6815105

Mustafa, S., Balkhy, H., and Gabere, M. N. (2018). Current treatment options and the role of peptides as potential therapeutic components for Middle East Respiratory Syndrome (MERS): a review. *J. Infect. Public Health* 11, 9–17. doi: 10.1016/j.jiph.2017.08.009

Nakagawa, K., Lokugamage, K. G., and Makino, S. (2016). "Viral and cellular mRNA translation in coronavirus-infected cells," in *Advances in Virus Research*, Vol. 96 (Cambridge, MA: Academic Press Inc.), 165–192. doi: 10.1016/bs.aivir.2016.08.001165

Nambiar, A., Heflin, M. E., Liu, S., Maslov, S., Hopkins, M., and Ritz, A. (2020). Transforming the Language of Life: Transformer Neural Networks for Protein Prediction Tasks. *bioRxiv*. 06.15.153643, (2020). doi: 10.1101/2020.06.15.153643

Naz, K., Naz, A., Ashraf, S. T., Rizwan, M., Ahmad, J., Baumbach, J., et al. (2019). PanRV: pangenome-reverse vaccinology approach for identifications of potential vaccine candidates in microbial pangenome. *BMC Bioinformatics* 20, 1–10. doi: 10.1186/s12859-019-2713-9

Oany, A. R., Al Emran, A., and Jyoti, T. (2014). Design of an epitope-based peptide vaccine against spike protein of human coronavirus: an *in silico* approach. *Drug Des. Devel. Ther.* 8, 1139–1149. doi: 10.2147/DDDT.S67861

Ong, E., Wang, H., Wong, M. U., Seetharaman, M., Valdez, N., and He, Y. (2020a). Vaxign-ML: supervised machine learning reverse vaccinology model for improved prediction of bacterial protective antigens. *Bioinformatics*36, 1–7. doi: 10.1093/bioinformatics/btaa119

Ong, E., Wong, M. U., Huffman, A., and He, Y. (2020b). COVID-19 coronavirus vaccine design using reverse vaccinology and machine learning. *bioRxiv [Preprint]*. doi: 10.1101/2020.03.20.000141

Pagadala, N. S., Syed, K., and Tuszynski, J. (2017). Software for molecular docking: a review. *Biophys. Rev.* 9, 91–102. doi: 10.1007/s12551-016-0247-1

Park, S. J., Kim, Y. G., and Park, H. J. (2011). Identification of rna pseudoknot-binding ligand that inhibits the - 1 ribosomal frameshifting of SARS-coronavirus by structure-based virtual screening. *J. Am. Chem. Soc.* 133, 10094–10100. doi: 10.1021/ja1098325

Pazhouhandeh, M., M.-Sahraian, A., Siadat, S. D., Fateh, A., Vaziri, F., Tabrizi, F., et al. (2018). A systems medicine approach reveals disordered immune system and lipid metabolism in multiple sclerosis patients. *Clin. Exp. Immunol.* 192, 18–32. doi: 10.1111/cei.13087

Pesciullesi, G., Schwaller, P., Laino, T., and and, J.-Reymond, L. (2020). *Carbohydrate transformer: predicting regio- and stereoselective reactions using transfer learning. ChemRxiv [Preprint]*. doi: 10.26434/chemrxiv.11935635

Pillaiyar, T., Meenakshisundaram, S., and Manickam, M. (2020). Recent discovery and development of inhibitors targeting coronaviruses. *Drug Discovery Today*. 5, 668–688. doi: 10.1016/j.drudis.2020.01.015

Plant, E. P., and Dinman, J. D. (2008). The role of programmed-1 ribosomal frameshifting in coronavirus propagation. *Front. Biosci.* 13, 4873–4881. doi: 10.2741/3046

Plant, H., Stacey, C., Tiong-Yip, C. L., Walsh, J., Yu, Q., and Rich, K. (2015). High-throughput hit screening cascade to identify respiratory syncytial virus (RSV) inhibitors. *J. Biomol. Screen.* 20, 597–605. doi: 10.1177/1087057115569428

Pollastri, G., Przybylski, D., Rost, B., and Baldi, P. (2002). Improving the prediction of protein secondary structure in three and eight classes using recurrent neural networks and profiles. *Proteins Struct. Funct. Genet.* 47, 228–235. doi: 10.1002/prot.10082

Prachar, M., Justesen, S., Steen-Jensen, D. B., Winther, O., and Bagger, F. O. (2020). COVID-19 vaccine candidates: prediction and validation of 174 SARS-CoV-2 epitopes. *bioRxiv [Preprint]*. doi: 10.1101/2020.03.20.000794

Prompetchara, E., Ketloy, C., and Palaga, T. (2020). Immune responses in COVID-19 and potential vaccines: Lessons learned from SARS and MERS epidemic. *Asian Pacific J. Allergy Immunol.* 38, 1–9. doi: 10.12932/AP-200220-0772

Rahman, M. S., Rahman, M. K., Saha, S., Kaykobad, M., and Rahman, M. S. (2019). Antigenic: an improved prediction model of protective antigens. *Artif. Intell. Med.* 94, 28–41. doi: 10.1016/j.artmed.2018.12.010

Rangan, R., Zheludev, I. N., and Das, R. (2020). RNA genome conservation and secondary structure in SARS-CoV-2 and SARS-related viruses. *bioRxiv [Preprint]*. doi: 10.1101/2020.03.27.012906

Rappuoli, R. (2000). Reverse vaccinology rino rappuoli. *Curr. Opin. Microbiol.* 3, 445–450. doi: 10.1016/S1369-5274(00)00119-3

Rasmussen, L., Maddox, C., Moore, B. P., Severson, W., and White, E. L. (2011). A high-throughput screening strategy to overcome virus instability. *Assay Drug Dev Technol.* 9, 184–190. doi: 10.1089/adt.2010.0298

Redka, D. S., MacKinnon, S. S., Landon, M., Windemuth, A., Kurji, N., and Shahani, V. (2020). PolypharmDB, a Deep Learning-Based Resource, Quickly Identifies Repurposed Drug Candidates for COVID-19. *ChemRxiv [Preprint]* doi: 10.26434/chemrxiv.12071271.v1

Richardson, P., Griffin, I., Tucker, C., Smith, D., Oechsle, O., Phelan, A., et al. (2020). Baricitinib as potential treatment for 2019-nCoV acute respiratory disease. *Lancet* 395, e30–e31, 15. doi: 10.1016/S0140-6736(20)30304-4

Salem, M., Khormali, A., Arshadi, A. K., Webb, J., and Yuan, S.-J. (2020). Transcreen: transfer learning on graph-based anti-cancer virtual screening model. *Big Data Cogn. Comput.* 4:16. doi: 10.3390/bdcc4030016

Sarkar, B., Ullah, M. A., Johora, F. T., Taniya, M. A., and Araf, Y. (2020). The essential facts of wuhan novel coronavirus outbreak in china and epitope-based vaccine designing against COVID-19. *bioRxiv [Preprint]*. doi: 10.1101/2020.02.05.935072

Scheller, J., Garbers, C., and Rose-John, S. (2014). Interleukin-6: From basic biology to selective blockade of pro-inflammatory activities. *Sem. Immunol.* 26, 2–12. doi: 10.1016/j.smim.2013.11.002

Senior, A. W., Evans, R., Jumper, J., Kirkpatrick, J., Sifre, L., Green, T., et al. (2020). Improved protein structure prediction using potentials from deep learning. *Nature* 577, 706–710. doi: 10.1038/s41586-019-1923-7

Shen, L., Niu, J., Wang, C., Huang, B., Wang, W., Zhu, N., et al. (2019). High-throughput screening and identification of potent broad-spectrum inhibitors of coronaviruses. *J. Virol.* doi: 10.1128/JVI.00023-19

Sher, G., Zhi, D., and Zhang, S. (2017). DRREP: deep ridge regressed epitope predictor. *BMC Genomics* 18:676. doi: 10.1186/s12864-017-4024-8

Shin, B., Park, S., Kang, K., and Ho, J. C. (2019). Self-attention based molecule representation for predicting drug-target interaction. *arXiv [Preprint] arXiv:*1908.06760.

Shoichet, B. K. (2004). Virtual screening of chemical libraries. *Nature* 432, 862–865. doi: 10.1038/nature03197

Shukla, P., Khandelwal, R., Sharma, D., Dhar, A., Nayarisseri, A., and Singh, S. K. (2019). Virtual screening of IL-6 inhibitors for idiopathic arthritis. *Bioinformation* 15, 121–130. doi: 10.6026/97320630015121

Simonovsky, M., and Komodakis, N. (2018). "GraphVAE: towards generation of small graphs using variational autoencoders," in *International Conference on Artificial Neural Networks* (Cham: Springer), 412–422. doi: 10.1007/978-3-030-01418-6_41

Smith, M., and Smith, J. C. (2020). Repurposing therapeutics for COVID-19: supercomputer-based docking to the SARS-CoV-2 viral spike protein and viral spike protein-human ACE2 interface. *ChemRxiv [Preprint]*. doi: 10.26434/chemrxiv.11871402.v4

Soria-Guerra, R. E., Nieto-Gomez, R., Govea-Alonso, D. O., and Rosales-Mendoza, S. (2015). An overview of bioinformatics tools for epitope prediction: Implications on vaccine development. *J. Biomed. Inform.* 53, 405–414. doi: 10.1016/j.jbi.2014.11.003

Stammler, S. N., Cao, S., Chen, S. J., and Giedroc, D. (2011). A conserved RNA pseudoknot in a putative molecular switch domain of the 3′-untranslated region of coronaviruses is only marginally stable. *RNA* 17, 1747–1759. doi: 10.1261/rna.2816711

Stebbing, J., Phelan, A., Griffin, I., Tucker, C., Oechsle, O., Smith, D., et al. (2020). COVID-19: combining antiviral and anti-inflammatory treatments. *The Lancet Infectious Diseases* 20, 400–402. doi: 10.1016/S1473-3099(20)30132-8

Sun, Y., Liang, D., Wang, X., and Tang, X. (2020). *DeepID3: Face Recognition with Very Deep Neural Networks*. Available online at: http://arxiv.org/abs/1502.00873 (accessed April 26, 2020).

Tanaka, T., Narazaki, M., and Kishimoto, T. (2016). Immunotherapeutic implications of IL-6 blockade for cytokine storm. *Immunotherapy* 8, 959–970. doi: 10.2217/imt-2016-0020

Tang, B., He, F., Liu, D., Fang, M., Wu, Z., and Xu, D. (2020). AI-aided design of novel targeted covalent inhibitors against SARS-CoV-2. *bioRxiv [Preprint]*. doi: 10.1101/2020.03.03.972133

Tilocca, B., Soggiu, A., Sanguinetti, M., Musella, V., Britti, D., Bonizzi, L., et al. (2020). Comparative computational analysis of SARS-CoV-2 nucleocapsid protein epitopes in taxonomically related coronaviruses. *Microbes Infect.* 22, 188–194. doi: 10.1016/j.micinf.2020.04.002

Ton, A.-T., Gentile, F., Hsing, M., Ban, F., and Cherkasov, A. (2020). Rapid identification of potential inhibitors of SARS-CoV-2 main protease by deep docking of 1.3 billion compounds. *Mol. Inform.* 39:202000028. doi: 10.1002/minf.202000028

Torng, W., and Altman, R. B. (2019). Graph convolutional neural networks for predicting drug-target interactions. *J. Chem. Inf. Model.* 59, 4131–4149. doi: 10.1021/acs.jcim.9b00628

Touret, F., Gilles, M., Barral, K., Nougairède, A., Decroly, E., de Lamballerie, X., et al. (2020). In vitro screening of a FDA approved chemical library reveals potential inhibitors of SARS-CoV-2 replication. *bioRxiv [Preprint]*. doi: 10.1101/2020.04.03.023846

Toxicology, EPA's National Center for Computational (2018). *ToxCast Database (invitroDB). The United States Environmental Protection Agency's Center for Computational Toxicology and Exposure. Dataset*. doi: 10.23645/epacomptox.6062623.v5

Tran, N. H., Qiao, R., Xin, L., Chen, X., Shan, B., and Li, M. (2019). Personalized deep learning of individual immunopeptidomes to identify neoantigens for cancer vaccines. *bioRxiv [Preprint]*. doi: 10.1101/620468

Vaishnav, N., Gupta, A., Paul, S., and John, G. J. (2015). Overview of computational vaccinology: vaccine development through information technology. *J. Appl. Genet.* 56, 381–391. doi: 10.1007/s13353-014-0265-2

Vaswani, A., Brain, G., Shazeer, N., Parmar, N., Uszkoreit, J., Jones, L., et al. (2017). "Attention is all you need," in *31st Conference Neural Infection Processing System (NIPS 2017)*.

Wallach, I., Dzamba, M., and Heifets, A. (2020). *AtomNet: A Deep Convolutional Neural Network for Bioactivity Prediction in Structure-based Drug Discovery*. Available onlion at: http://arxiv.org/abs/1510.02855 (accessed April 22, 2020).

Wan, Y., Shang, J., Sun, S., Tai, W., Chen, J., Geng, Q., et al. (2019). Molecular mechanism for antibody-dependent enhancement of coronavirus entry. *J. Virol.* 94, 1–15. doi: 10.1128/JVI.02015-19

Wang, D., Liu, W., Shen, Z., Jiang, L., Wang, J., Li, S., et al. (2020). Deep learning based drug metabolites prediction. *Front. Pharmacol.* 10:1586. doi: 10.3389/fphar.2019.01586

Wang, G., Li, X., and Wang, Z. (2015). APD3: the antimicrobial peptide database as a tool for research and education. *Nucleic Acids Res.* 44, 1087–1093. doi: 10.1093/nar/gkv1278

Weiss, K., Khoshgoftaar, T. M., and Wang, D. D. (2016). A survey of transfer learning. *Big Data J.* 3:9. doi: 10.1186/s40537-016-0043-6

Worldometer (2020). *Coronavirus Cases. Worldometer*. Available online at: https://www.worldometers.info/coronavirus/coronavirus-cases/#daily-cases (accessed April 27, 2020).

Wu, C., Liu, Y., Yang, Y., Zhang, P., Zhong, W., Wang, Y., et al. (2020). Analysis of therapeutic targets for SARS-CoV-2 and discovery of potential drugs by computational methods. *Acta Pharm. Sin. B.* 10, 766–788. doi: 10.1016/j.apsb.2020.02.008

Wu, J., Wang, W., Zhang, J., Zhou, B., Zhao, W., Su, Z., et al. (2019). DeepHLApan: a deep learning approach for neoantigen prediction considering both HLA-peptide binding and immunogenicity. *Front. Immunol.* 10:2559. doi: 10.3389/fimmu.2019.02559

Xia, S., Xu, W., Wang, Q., Wang, C., Hua, C., Li, W., et al. (2020). *Peptide-Based Membrane Fusion Inhibitors Targeting HCoV-229E Spike Protein HR1 and HR2 Domains. mdpi.com*. Available online at: https://www.mdpi.com/1422-0067/19/2/487 (accessed April 28, 2020).

Xiang, Z., and He, Y. (2009). Vaxign: a web-based vaccine target design program for reverse vaccinology. *Procedia Vaccinol.* 1, 23–29. doi: 10.1016/j.provac.2009.07.005

Yang, D., and Leibowitz, J. L. (2015). The structure and functions of coronavirus genomic 3' and 5' ends. *Virus Research* 206, 120–133. doi: 10.1016/j.virusres.2015.02.025

Yu, W., and Mackerell, A. D. (2017). "Computer-aided drug design methods," in *Methods in Molecular Biology, Vol. 1520,* ed P. Sass (New York, NY: Humana Press Inc.), 85–106. doi: 10.1007/978-1-4939-6634-9_5

Zhai, S., Chang, K., Zhang, R., and Zhang, Z. (2016). DeepIntent: Learning attentions for online advertising with recurrent neural networks KDD'16. in *Proceedings of the 22nd ACM SIGKDD International Conference on Knowledge Discovery and Data Mining* (New York, NY: Association for Computing Machinery), 1295–1304.

Zhang, C., Wu, Z., Li, J.-W., Zhao, H., and Wang, G.-Q. (2020). The cytokine release syndrome (CRS) of severe COVID-19 and Interleukin-6 receptor (IL-6R) antagonist Tocilizumab may be the key to reduce the mortality. *Int. J. Antimicrob. Agents* 55:105954. doi: 10.1016/j.ijantimicag.2020.105954

Zhang, H., Saravanan, K. M., Yang, Y., and Hossain, T. (2020). Deep learning based drug screening for novel coronavirus 2019-nCov. *Prepr* 19, 1–17. doi: 10.20944/preprints202002.0061.v1

Zhang, L., Lin, D., Sun, X., Curth, U., Drosten, C., Sauerhering, L., et al. (2020). Crystal structure of SARS-CoV-2 main protease provides a basis for design of improved α-ketoamide inhibitors. *Science* 368:eabb3405. doi: 10.1126/science.abb3405

Zhavoronkov, A., Aladinskiy, V., Zhebrak, A., Zagribelnyy, B., Terentiev, V., Bezrukov, D. S., et al. (2020). Potential 2019-nCoV 3C-like protease inhibitors designed using generative deep learning approaches Potential COVID-19 3C-like protease inhibitors designed using generative deep learning approaches. *Insilico Med. Hong Kong Ltd A* 307:E1. doi: 10.26434/chemrxiv.829102.v1

Zhavoronkov, A., Ivanenkov, Y. A., Aliper, A., Veselov, M. S., Aladinskiy, V. A., Aladinskaya, A. V., et al. (2019). Deep learning enables rapid identification of potent DDR1 kinase inhibitors. *Nat. Biotechnol.* 37, 1038–1040. doi: 10.1038/s41587-019-0224-x

Zheng, C., Yu, W., Xie, F., Chen, W., Mercado, C., Sy, L. S., et al. (2019). The use of natural language processing to identify Tdap-related local reactions at five health care systems in the Vaccine Safety Datalink. *Int. J. Med. Inform.* 127, 27–34. doi: 10.1016/j.ijmedinf.2019.04.009

Zhong, F., Xing, J., Li, X., Liu, X., Fu, Z., Xiong, Z., et al. (2018). Artificial intelligence in drug design. *Sci. China Life Sci.* 61, 1191–1204. doi: 10.1007/s11427-018-9342-2

Zhou, H., Gao, M., and Skolnick, J. (2015). Comprehensive prediction of drug-protein interactions and side effects for the human proteome. *Sci. Rep.* 5:11090. doi: 10.1038/srep11090

Zhou, P., Yang, X.-L., Wang, X.-G., Hu, B., Zhang, L., Zhang, W., et al. (2020). A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature* 579, 270–273. doi: 10.1038/s41586-020-2012-7

Zhou, Y., Hou, Y., Shen, J., Huang, Y., Martin, W., and Cheng, F. (2020). Network-based drug repurposing for novel coronavirus 2019-nCoV/SARS-CoV-2. *Cell Discov.* 6:14. doi: 10.1038/s41421-020-0153-3

Conflict of Interest: EC, ED-C, and BK were employed by the company A2A Pharmaceuticals. SC-T was employed by the company Atomwise Inc.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 19 August 2020
doi: 10.3389/fmicb.2020.02014

The Strengths of Scanning Electron Microscopy in Deciphering SARS-CoV-2 Infectious Cycle

*Djamal Brahim Belhaouari[1], Anthony Fontanini[2], Jean-Pierre Baudoin[1], Gabriel Haddad[1], Marion Le Bideau[1], Jacques Yaacoub Bou Khalil[2], Didier Raoult[1] and Bernard La Scola[1,2]**

[1] Microbes, Evolution, Phylogeny and Infection (MEPHI), UM63, Institut de Recherche pour le Développement (IRD), Assistance Publique – Hôpitaux de Marseille (AP-HM), Aix-Marseille University, Marseille, France, [2] IHU Méditerranée Infection, Marseille, France

Electron microscopy is a powerful tool in the field of microbiology. It has played a key role in the rapid diagnosis of viruses in patient samples and has contributed significantly to the clarification of virus structure and function, helping to guide the public health response to emerging viral infections. In the present study, we used scanning electron microscopy (SEM) to study the infectious cycle of SARS-CoV-2 in Vero E6 cells and we controlled some key findings by classical transmission electronic microscopy (TEM). The replication cycle of the virus was followed from 1 to 36 h post-infection. Our results revealed that SARS-CoV-2 infected the cells through membrane fusion. Particles are formed in the peri-nuclear region from a budding of the endoplasmic reticulum-Golgi apparatus complex into morphogenesis matrix vesicae. New SARS-CoV-2 particles were expelled from the cells, through cell lysis or by fusion of virus containing vacuoles with the cell plasma membrane. Overall, this cycle is highly comparable to that of SARS-CoV. By providing a detailed and complete SARS-CoV-2 infectious cycle, SEM proves to be a very rapid and efficient tool compared to classical TEM.

Keywords: SARS-CoV-2, infectious cycle, Vero E6 cells, scanning electron microscopy, Coronavirus

OPEN ACCESS

Edited by:
Fatah Kashanchi,
George Mason University,
United States

Reviewed by:
Maria C. Silva,
Federal University of ABC, Brazil
Joel D. Baines,
Louisiana State University,
United States

***Correspondence:**
Bernard La Scola
bernard.la-scola@univ-amu.fr

Specialty section:
This article was submitted to Virology, a section of the journal Frontiers in Microbiology

Received: *14 May 2020*
Accepted: *30 July 2020*
Published: *19 August 2020*

Citation:
Brahim Belhaouari D, Fontanini A, Baudoin J-P, Haddad G, Le Bideau M, Bou Khalil JY, Raoult D and La Scola B (2020) The Strengths of Scanning Electron Microscopy in Deciphering SARS-CoV-2 Infectious Cycle. Front. Microbiol. 11:2014. doi: 10.3389/fmicb.2020.02014

INTRODUCTION

The SARS-CoV-2 (COVID-19) outbreak started in late December 2019 in China and has since reached a global pandemic (Zhu et al., 2020), leading to a worldwide battle against COVID-19. SARS-CoV-2 is a novel β-coronavirus belonging to the sarbecovirus subgenus of Coronaviridae family (Schoeman and Fielding, 2019; Zhu et al., 2020). Coronaviruses are enveloped viruses with a positive sense, single-stranded RNA genome (Schoeman and Fielding, 2019). One of the first methods used for coronaviruses detection was electron microscopy (EM), which has been a reliable tool for the classification of viruses according to their ultra-structure (Hazelton and Gelderblom, 2003; Curry et al., 2006). The characteristic morphology of crown-like structures detected by EM explains the name of Coronaviridae family (Golding et al., 2016) observed as widely spaced club-shaped projections surrounding the virus envelope, thus forming a crown aspect in negative staining protocols (Almeida and Tyrrell, 1967; Oshiro et al., 1971). Coronaviruses have the largest genomes among RNA viruses, with genome sizes ranging from 26 to 32 kb in length. These viruses primarily infect birds and mammals, and can also infect humans, causing respiratory and enteric diseases, such as upper respiratory tract infections and lower respiratory tract infections (bronchitis, pneumonia, and severe acute respiratory syndrome (SARS). EM is a powerful tool in

the field of microbiology, because of its resolution power as compared to light microscopy (Koster and Klumperman, 2003). EM contributed significantly to the clarification of viruses structure and function and has played a key role in the rapid diagnosis of viruses in various samples (Goldsmith and Miller, 2009). The ability of EM to detect unknown and unsuspected organisms has made it a suitable tool to guide the public health response during previous outbreaks. Transmission electron microscopy (TEM) was extensively used to describe the morphology or the morphogenesis of SARS-CoV (Ng et al., 2003; Qinfen et al., 2004), MERS-CoV (Kim et al., 2016; Park et al., 2016; Alsaad et al., 2018) or, more recently, SARS-CoV-2 (Caly et al., 2020; Colson et al., 2020; Kim et al., 2020; Zhu et al., 2020).

Scanning electron microscopy (SEM) is another powerful tool for microbiological research and diagnosis of infectious diseases (Golding et al., 2016). We already demonstrated its strengths for ultra-rapid microscope imaging of SARS-CoV-2 when pandemic first reached France (Colson et al., 2020). Here, we used SEM for its capacity to rapidly screen SARS-CoV-2-infected Vero cells in resin ultra-thin sections, allowing the ultrastructural detailed analysis of SARS-CoV-2 throughout the whole infectious cycle.

MATERIALS AND METHODS

Cell Culture-Virus Infectious Cycle

Vero E6 cells were grown to monolayer in 25 cm^2 culture flasks in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum for 2–3 days at 37°C. For the viral infection cycle, the culture medium were removed, and the cells were inoculated with SARS-CoV-2 at a multiplicity of infection (MOI) of 1. After incubation at 37°C for 45 min, the supernatant was removed. This marked time 0 (H0). For later time points, infected cells were incubated at 37°C in medium culture. Post-infection time points were: 1, 2, 3, 4, 5, 6, 12, 24, and 36 h post infection. For each time point, infected cells were detached by using 500 µl of trypsin and pelleted by centrifugation at 500 × g for 10 min.

Scanning and Transmission Electron Microscopy

For electron microscopy infected Vero cells were fixed at least for 1 h with glutaraldehyde 2.5% in 0.1M sodium cacodylate buffer. For resin embedding, cells were washed three times with a mixture of 0.2M saccharose/0.1M sodium cacodylate. Cells were post-fixed for 1 h with 1% OsO4 diluted in 0.2M Potassium hexa-cyanoferrate (III) / 0.1M sodium cacodylate solution. After three 10 min washes with distilled water, the cells were gradually dehydrated with ethanol by successive 10 min baths in 30, 50, 70, 96, 100, and 100% ethanol. Substitution was achieved by successively placing the cells in 25, 50, and 75% Epon solutions for 15 min. Cells were placed for 1 h in 100% Epon solution and in fresh Epon 100% over-night under vacuum at room-temperature. Polymerization occured with cells in 100% fresh Epon for 72 h at 60°C. All solutions used above were 0.2 µm filtered. Ultrathin 70 nm sections were cut using a UC7 ultramicrotome (Leica) and placed on HR25 300 Mesh Copper/Rhodium grids (TAAB, United Kingdom). Sections were contrasted according to Reynolds (1963). For scanning electron microscopy (SEM), grids with sections were mounted on double-sided tape on glass slide for sequential observation of different time-points and they were platinum-coated with a MC1000 sputter coater (Hitachi) for 40 s at 10 mA. Electron micrographs were obtained on either SU5000 SEM (Hitachi High-Tech, HHT, Japan) operated between 7 and 10 kV accelerating voltage, in high-vacuum and observation mode (spot size 30), between 4.6 and 4.9 mm average working distance with BSE detector, and magnifications ranging from ×5,000 to ×100,000 or Tecnai G2 TEM (Thermo-Fischer/FEI) operated at 200 keV equipped with a 4096 × 4096 pixels resolution Eagle camera (FEI).

RESULTS

SARS-CoV-2 Cell Entry

At early post-infection time-point SARS-CoV-2 virions were detected by SEM and TEM, located at the surface of the cells (**Figure 1**). In those cells, we did not notice viral morphogenesis features, and particles were seen i) attached, with their corona spikes located between the particle and the plasma membrane (**Figures 1C,D**) or ii) less electron-dense, with the envelope fusing with the plasma membrane (**Figures 1E,F**). Endocytic vesicles with typical clathrin-coated pits were often observed below particles attached to the plasma membrane (**Figures 2E,F**). In these forming endocytic particles, rod-like amorphous material was present (**Figure 2**). This kind of material was also observed in clathrin-coated endocytic vesicles located more deeply in the cell cytoplasm (**Figures 2G,H**). We also observed in the cytoplasm of cells with particles at the plasma membrane electron-dense crescent-shaped intracellular structures (**Figures 2A–C**), that were also present in control, non-infected cells (**Supplementary Figure 1**). A very few virus-like particles were also observed inside the cells, in endoplasmic reticulum (ER)-derived peripheral canaliculi (not shown). From H1 to H5 SARS-CoV-2 virions were not detected in the ultra-thin sections.

From H12, SARS-CoV-2 virions were found attached to lyzed cells containing vacuoles filled with nascent particles (**Figure 3A**) or attached to cells containing mature SARS-CoV-2 particles with corona spikes located in small cytoplasmic vacuoles, between the nucleus and the cell periphery (**Figures 3B–D**) and also attached to cells without morphogenesis features (**Figures 1C,F**). From H18 onwards, most of the viruses attached to cell plasma membranes were seen in virus-producing-cells, which were lyzed or intact.

SARS-CoV-2 Morphogenesis

First, swollen nuclear membrane, endoplasmic reticulum (ER) and Golgi apparatus (GA) organelles were the most striking features of SARS-CoV-2 infected cells (**Figure 4**). Thick and distorted ER tubules were observed at peri-nuclear locations between the nucleus and the GA (**Figures 4C,D**), and also at peripheral locations below the plasma membrane, where the ER could be seen as zippered (**Figure 4G**). When intact, the GA was found at peri-nuclear locations, with Golgi stacks lying

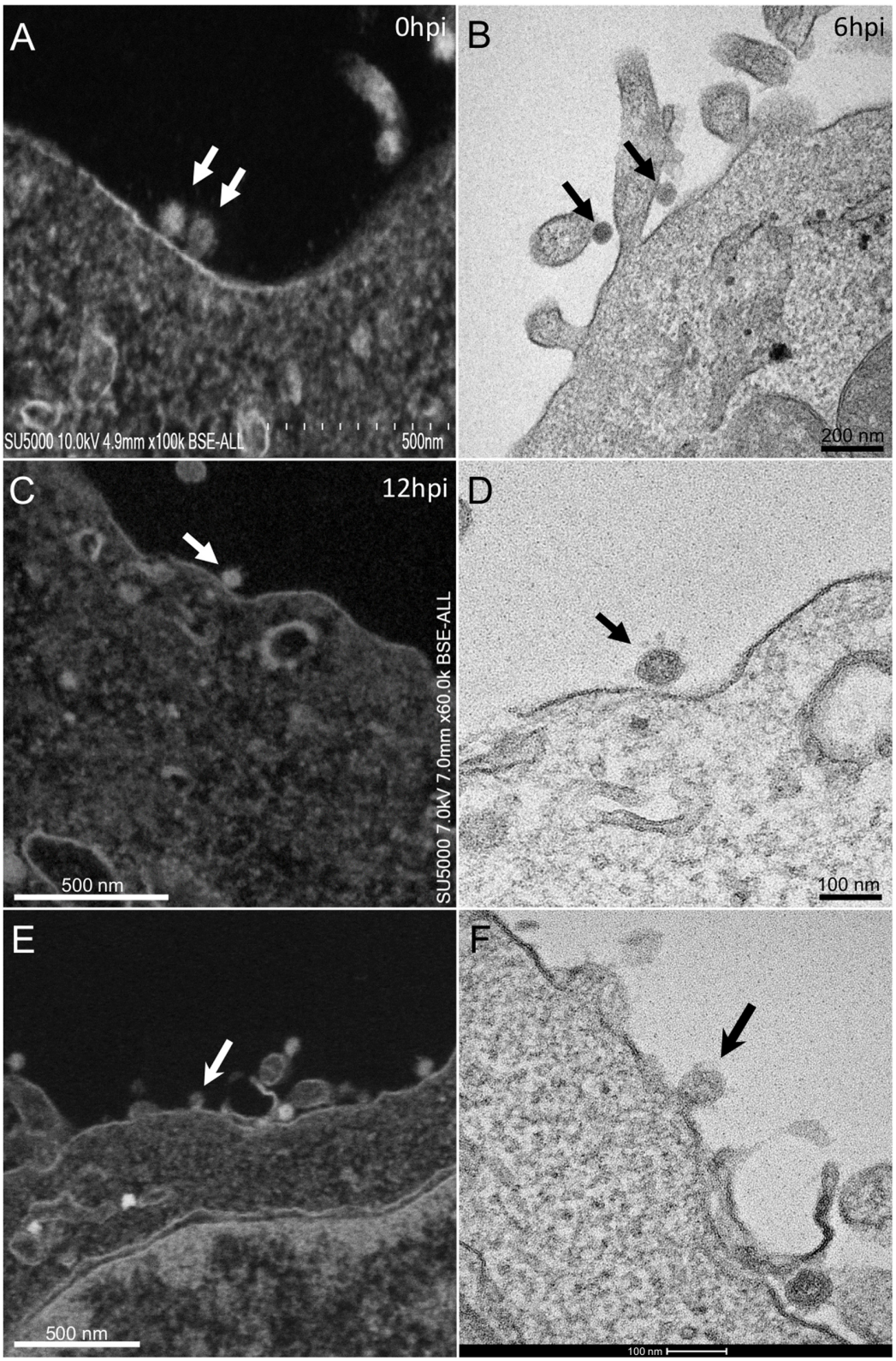


FIGURE 1 | SARS-CoV-2 infected Vero E6 cells At early post-infection time-point with virus **(A,B)** at the periphery of Vero E6 cells (arrows). **(C,D)** SEM **(C)** and TEM **(D)** views of the same cellular region with a SARS-CoV-2 particle (arrow) attached to the plasma membrane, the corona spikes of which are located between the particle and the cell plasma membrane. **(E,F)** SEM **(E)** and TEM **(F)** views of the same cellular region showing SARS-CoV-2 virus particles attached to the cell plasma membrane; one particle (arrow in **E,F**) is glued to the plasma membrane, fusing with the cell plasma membrane.

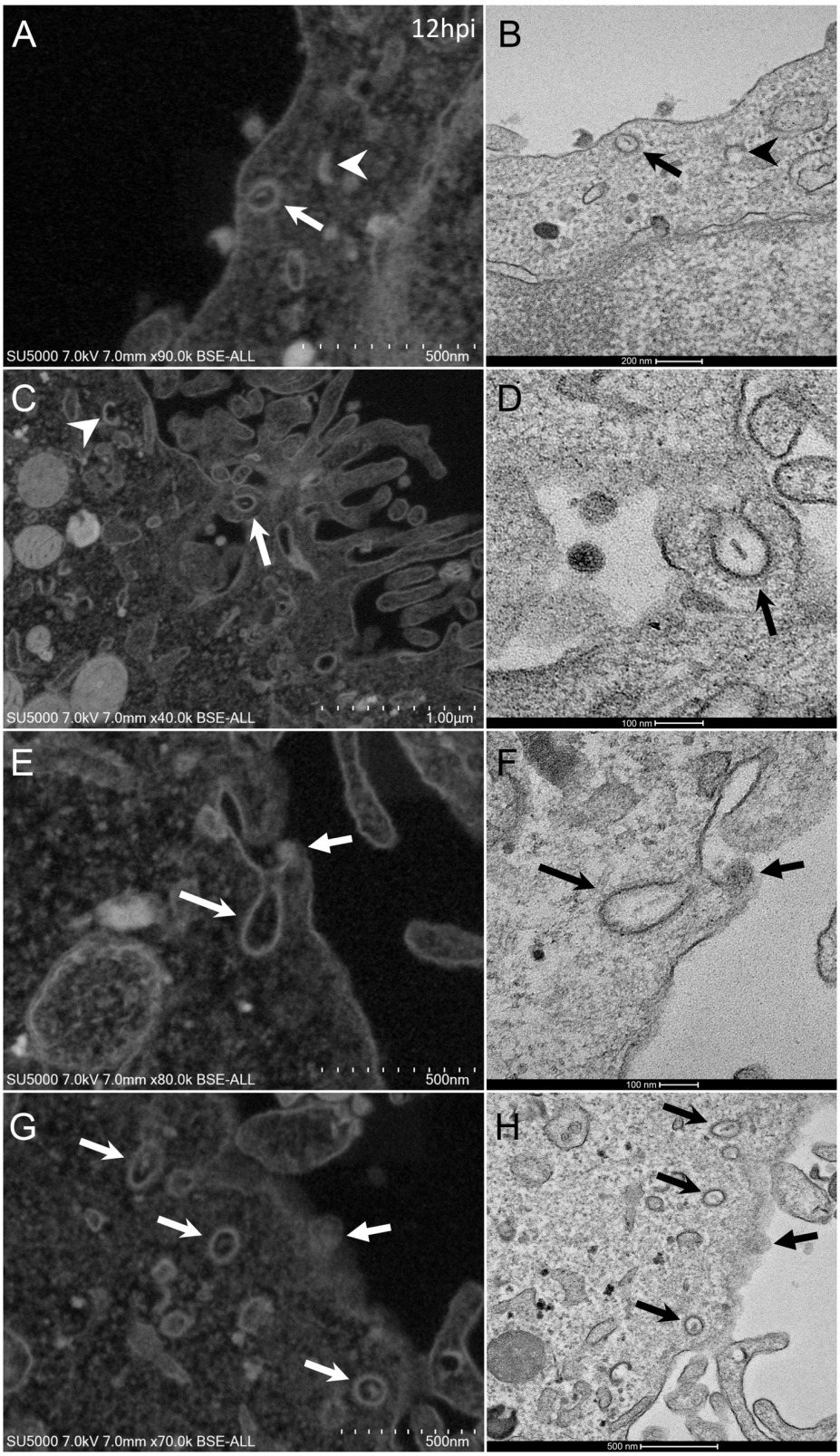


FIGURE 2 | SARS-CoV-2 infected Vero E6 cells with endocytic vesicles in the cytoplasm. **(A–F)** SEM **(A,C,E,G)** and TEM **(B,D,F,H)** views of the same cellular regions with clathrin-coated vesicles (arrows) containing rod-like amorphous material. Crescent-like electron-dense structures (arrowhead in **A**) were often depicted in infected cells cytoplasm. Solid arrows **(E–H)** point to glued SARS-CoV-2 particles on cells plasma membrane.

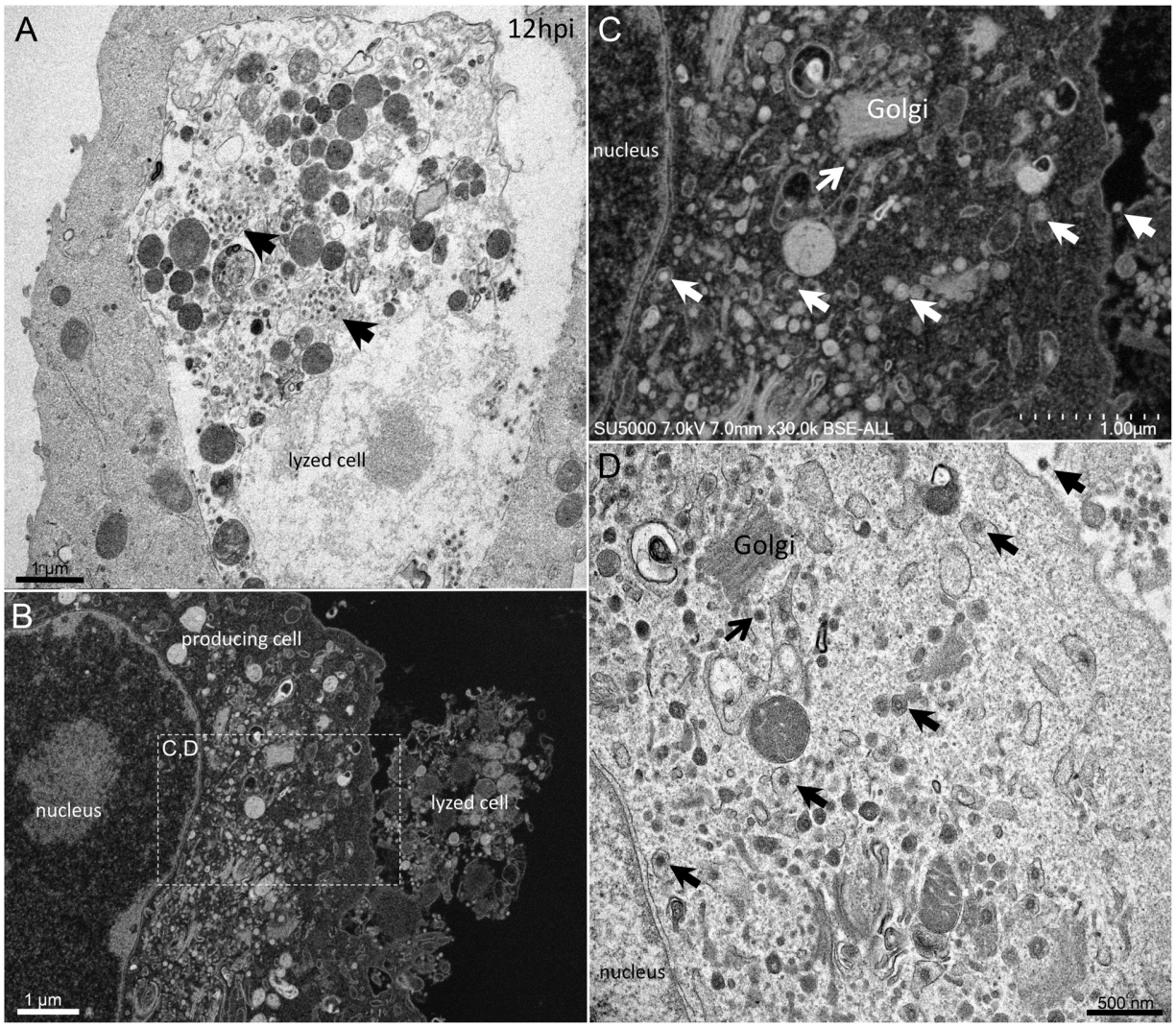


FIGURE 3 | SARS-CoV-2 infected Vero E6 cells at 12 h post-infection. **(A)** Cell in lytic process with vacuole filled with nascent particles (arrows). **(B)** producing-virus cell adjacent to lysed cell. **(C,D)** zooming on virus-producing cell shows mature SARS-CoV-2 particles with their corona spikes (arrows) in small cytoplasmic vacuoles between the nucleus and the cell periphery, observed by SEM **(C)** or TEM **(D)** in a cell. Extracellular particles attached to the plasma membrane (arrows).

parallel to the ER and the nuclear membranes (**Figures 4C,D**). As infection progressed, the GA was found budding between large ER tubules, resulting in multiple Golgi-derived nascent particles and a loss of intact GA stacks (**Figures 4A,B,F**). The extent of GA budding was variable from a cell to another, being generally proportional to its distance from the nucleus. At early infection stages, myelin-like membranes whorls were present at proximity of the Golgi apparatus (**Figure 4E**). These whorl types are probably not a typical feature of infected cells, as they were also observed in uninfected cells (**Supplementary Figure 1**). We noticed abundant mitochondria in apical regions of both non-infected and infected cells at all stages, around the ER and Golgi-rich regions (not shown).

Golgi-derived doughnut-like particles with a pronounced electron-opaque edge were observed at peri-nuclear locations (**Figures 5A,B**), dispersed into the cytoplasm, as well as entering vacuoles, which seemed to be derived from the ER (**Figures 5A,B**). Such forming virus morphogenesis matrix vesicae (VMMV) (Qinfen et al., 2004), filled with doughnut-like particles, were observed as open sacs, assembling next to the nucleus (**Figures 5A,B**), or closed sacs adjacent to or distant from the nucleus, in the cytoplasm or in the vacuoles (**Figures 5C,D**). Nascent particles were first observed at H12 in only a few cells (**Figure 3**), to a lesser extent than in more advanced times of infection. Doughnut-like particles were 70 ± 6 nm in diameter (n = 100), devoid of corona spikes. Their shape was not perfectly round when observed in the assembling opened sacs, and these particles could present filopodia-like protrusion (**Figure 5B**).

As the infection progressed, we observed an extensive network of membrane whorls, with large inter-membranous distances when compared to previous "small" membrane whorls (**Figure 6A**). These intermingled membranes were lying at the

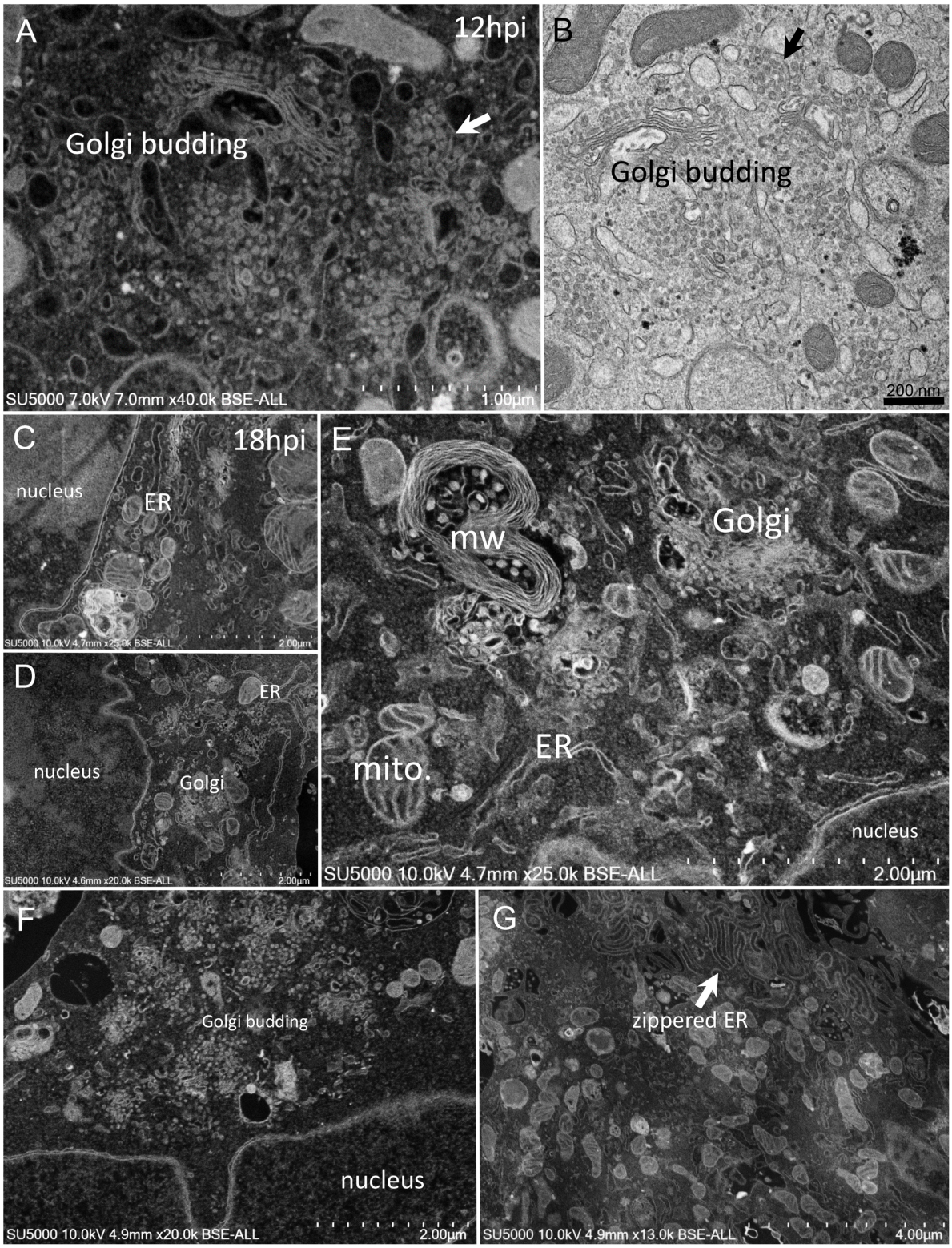


FIGURE 4 | SARS-CoV-2 infected Vero E6 cells at 12–18 h post-infection. **(A,B)** SEM **(A)** and TEM **(B)** views of the same cell region showing extreme Golgi apparatus budding as well as a vacuole filled with nascent particles close to the Golgi apparatus. **(C,D)** Thick and distorted endoplasmic reticulum (ER) tubules were observed by SEM at peri-nuclear location and at the cells periphery. **(E)** SEM view of the extensive enlargement and budding of the ER and Golgi apparatus, as well as a myelin-like membranes whorl (mw) close to the Golgi apparatus and mitochondria (mito.) in the perinuclear region. **(F)** SEM low-magnification view of Golgi budding between a nucleus and the peripheral plasma membrane. **(G)** SEM image of zippered endoplasmic reticulum (ER) at apical location (arrow).

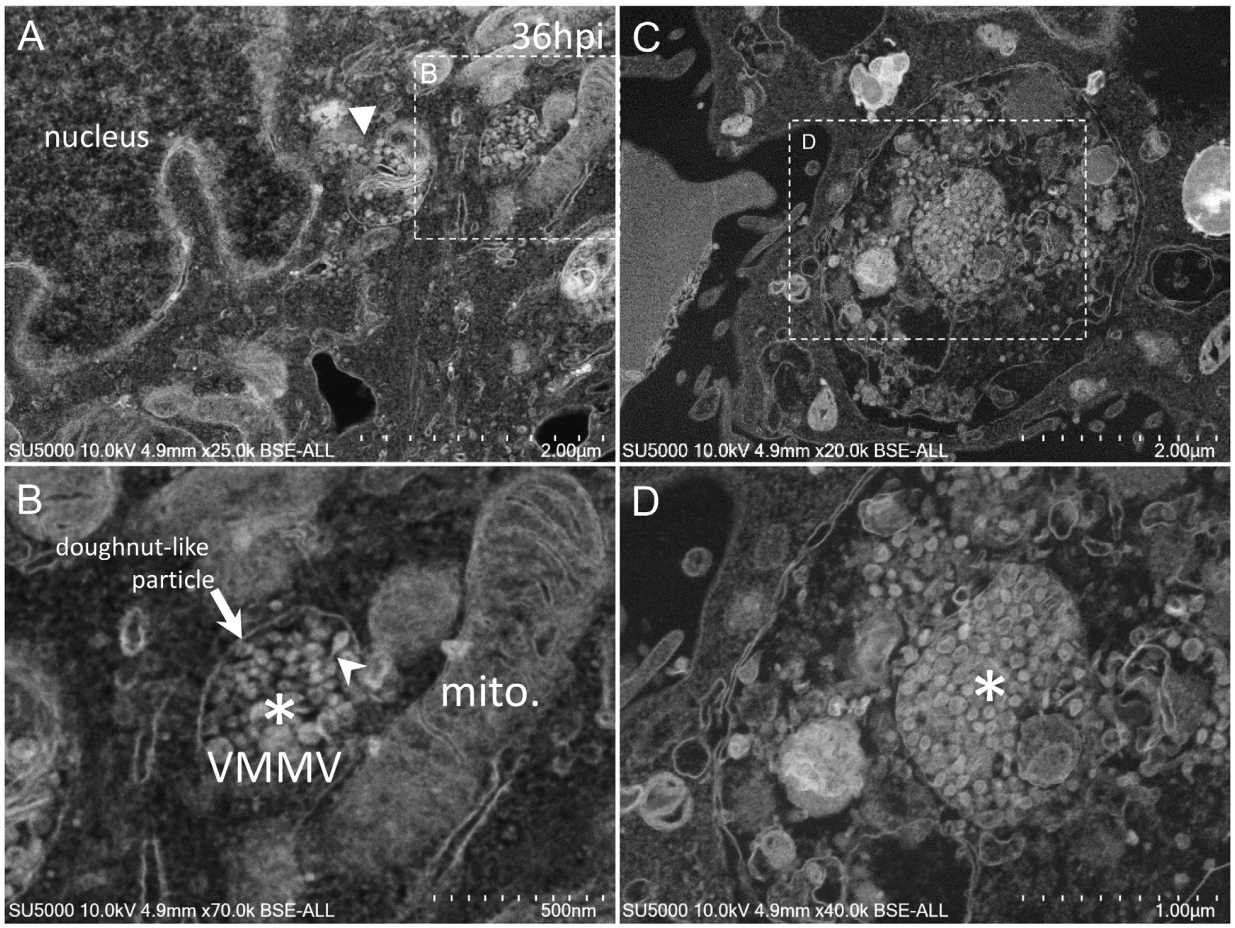


FIGURE 5 | SARS-CoV-2 infected Vero E6 cells at 36 h post-infection. **(A,B)** SEM views of doughnut-like particles (arrow) with a pronounced electron-opaque edge observed at peri-nuclear locations dispersed into the cytoplasm (solid arrowhead), or within vacuoles, which seemed derived from the ER, forming virus morphogenesis matrix vesicae (VMMV). VMMV were observed as opened sacs (asterisk in **B**), assembling next to the nucleus **(A,B)**. Protrusions of the doughnut-like particles could be observed in the VMMV (arrowhead in **B**). **(C,D)** VMMV observed as a closed sac (asterisk in **D**) in a larger vacuole in the cytoplasm. **(A–D)** SEM images.

level of concave nuclear indentations. Nascent virions could be found mixed with such membranes whorls in large bags (**Figure 6B**). The appearance of the VMMV was variable, with nascent particles located in more or less large vacuoles, containing more or less electron-dense material (**Figures 6C–F**). The electron-density of these vacuoles filled with nascent particles was correlated with the heterogeneity of these vacuoles: electron-dense virions-filled vacuoles were homogenous (**Figures 6C,D**), while electron-lucent vacuoles contained virions particles as well as heterogeneous materials such as membranes, or distorted compartments (**Figures 6E,F**). VMMV located below the plasma membrane were frequently seen translucent, with well-individualized virions particles (**Figure 6F**). Particles could arrange as circular chains lying on the internal surface of the VMMV (**Figure 6D**).

We also noticed at nucleus margins of infected cells, round and empty objects with ± a punctate pattern at their center (**Supplementary Figures 2A–F**). These objects 100–120 nm in diameter were present at locations where the nuclear membrane was not clearly delineated, in contrast to adjacent regions where the nucleus double membrane was properly seen. These features could be observed in transverse (**Supplementary Figures 2A–E,G**) or in tangential sections of the nuclei (**Supplementary Figures 2F,H**), the objects being in the latter case, located in an electron-dense chromatin-like material, which we called nuclear matrix. These objects were also observed, to a lesser extent, in uninfected cells (**Supplementary Figure 3**).

SARS-CoV-2 Cell Exit

Mature SARS-CoV-2 particles 80 ± 7 nm in diameter (n = 100) were observed as spiky round to hexagonal electron-dense particles (**Figure 7**). Mature SARS-CoV-2 particles were observed at extra- and intra-cellular locations: in translucent VMMV or vacuoles of early-infected intact- cells (**Figure 6F**), and later found lying between cellular microvilli (**Figures 7A–C**), in vacuoles (**Figure 7G**), as well as on the surface of lysed cells (**Figures 7F,H**). Intracellular compartments filled with mature

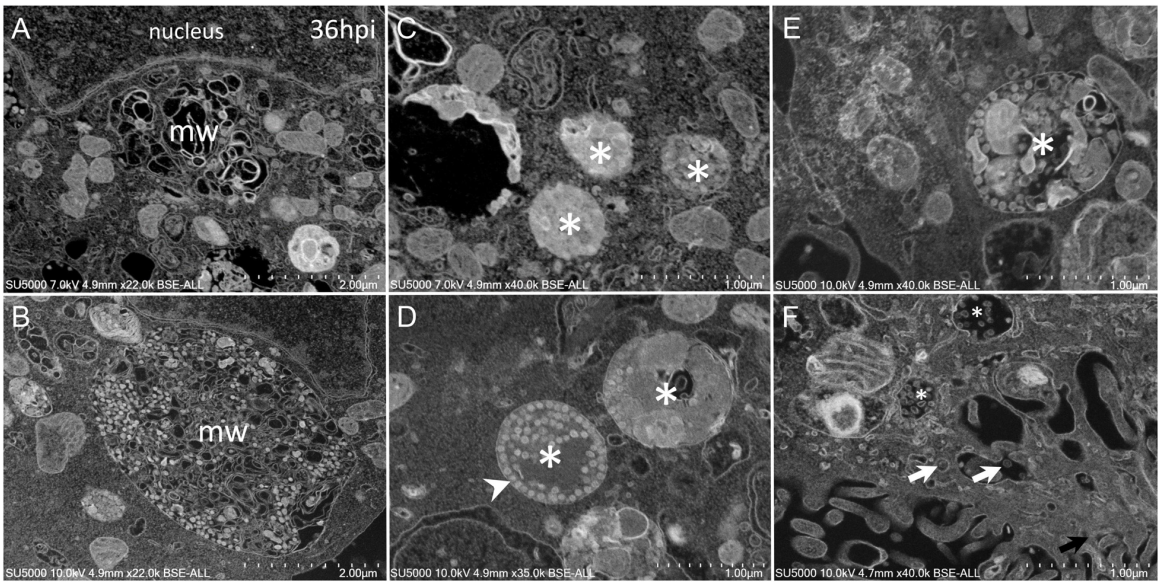


FIGURE 6 | SARS-CoV-2 infected Vero cells at 36 h post-infection **(A)** SEM depiction of an extensive peri-nuclear membrane whorls (mw) network, with larger inner-membranous distances as infection progresses. **(B)** Nascent virions particles (≈70 nm) observed by SEM mixed with membranes whorls in large bags. **(C–F)** SARS-CoV-2-morphogenesis matrix vesicae (asterisk) with different appearances, with nascent particles (≈70 nm) located in more or less large vacuoles, containing more or less electron-dense materials (asterisk). **(F)** Translucent VMMV/vacuoles with well-individualized virions particles (white arrows).

virions were observed channeling with the apical side of the Vero cells at the base of the microvilli (**Figures 7C,E**).

DISCUSSION

Our results show spiky round to hexagonal 80 nm in diameter electron-dense mature SARS-CoV-2 particles, similar to the previously described SARS-CoV-2 virions (Colson et al., 2020; Kim et al., 2020; Zhu et al., 2020). Previous analysis of ultrathin sections by TEM of SARS-CoV-2 infected- cells showed virus particles in inclusion bodies in human airway cells (Zhu et al., 2020), as well as in a wide range of intracellular organelles, especially in vesicles of Vero cells (Caly et al., 2020; Kim et al., 2020). Here, thanks to our SEM analysis, we were able to show the similarity between SARS-CoV-2 and SARS-CoV infectious cycles, with some exceptions.

In our experiments, At early post-infection time-point, SARS-CoV-2 virions were seen attached at cells plasma membrane. The absence of SARS-CoV-2 virions observation from H1 to H5 is probably related to the very brief binding of infectious particles to the cell surface, their rapid reduction once attached to the cells and a consecutive eclipse phase. From H12 post-infection, different cell profiles were seen: no producing-virus cells, virus-producing cells and lysed cells. This variability in the stage of infection is probably due to a desynchronized virus infection due to cells not being infected at the same time or to a low infectious titer of virus used to infect the cells. At H12, attached particles to cell plasma membranes were seen in non-virus-producing cells, probably corresponding to cells being infected by neo-synthesized virions produced by adjacent infected cells, and also in virus-producing cells with morphogenesis features. For this latter case, it is not known if virions attachment onto already infected cells would yield further productive cycles of replication.

Regarding SARS-CoV-2 cell entry process, particles were observed attached at cells plasma membrane, located at cells apical sides, similarly to SARS-CoV (Rossen et al., 1994). Full SARS-CoV-2 particle endocytosis was not observed, which is consistent with previous studies conducted on SARS-CoV particles infecting cells by membrane fusion (Oshiro et al., 1971; Ng et al., 2003; Qinfen et al., 2004). In fact, we observed a possible fusion of SARS-CoV-2 particles with the cells plasma membranes. Our images suggest that these attached particles were probably caught transferring their content inside the cell cytoplasm. The role of the clathrin endocytic vesicles containing amorphous material, as intermediate receptacles of SARS-CoV-2 genomic content after fusion of the particles with cells plasma membranes, is likely to be part of the SARS-CoV-2 nucleocapsid cell entry process.

Regarding SARS-CoV-2 morphogenesis, previous studies reported nuclear localization of SARS-CoV proteins or particles (Zhang et al., 2003; Qinfen et al., 2004; Yuan et al., 2005). Here, we did not observe SARS-CoV-2 particles inside the nucleus. We observed round objects in a nuclear matrix without distinctive membranous limits, in infected as well as in non-infected cells (**Supplementary Figures 2, 3**). These objects likely correspond to nuclear pore complexes according to morphology (round ± punctate pattern) and diameter (Bardina et al., 2009; Lin and Hoelz, 2019). The abundance of mitochondria next to the ER and GA budding regions, where SARS-CoV-2 morphogenesis occurred, could provide energy for viral multiplication (de Castro et al., 2013). It was assumed that doughnut-shaped

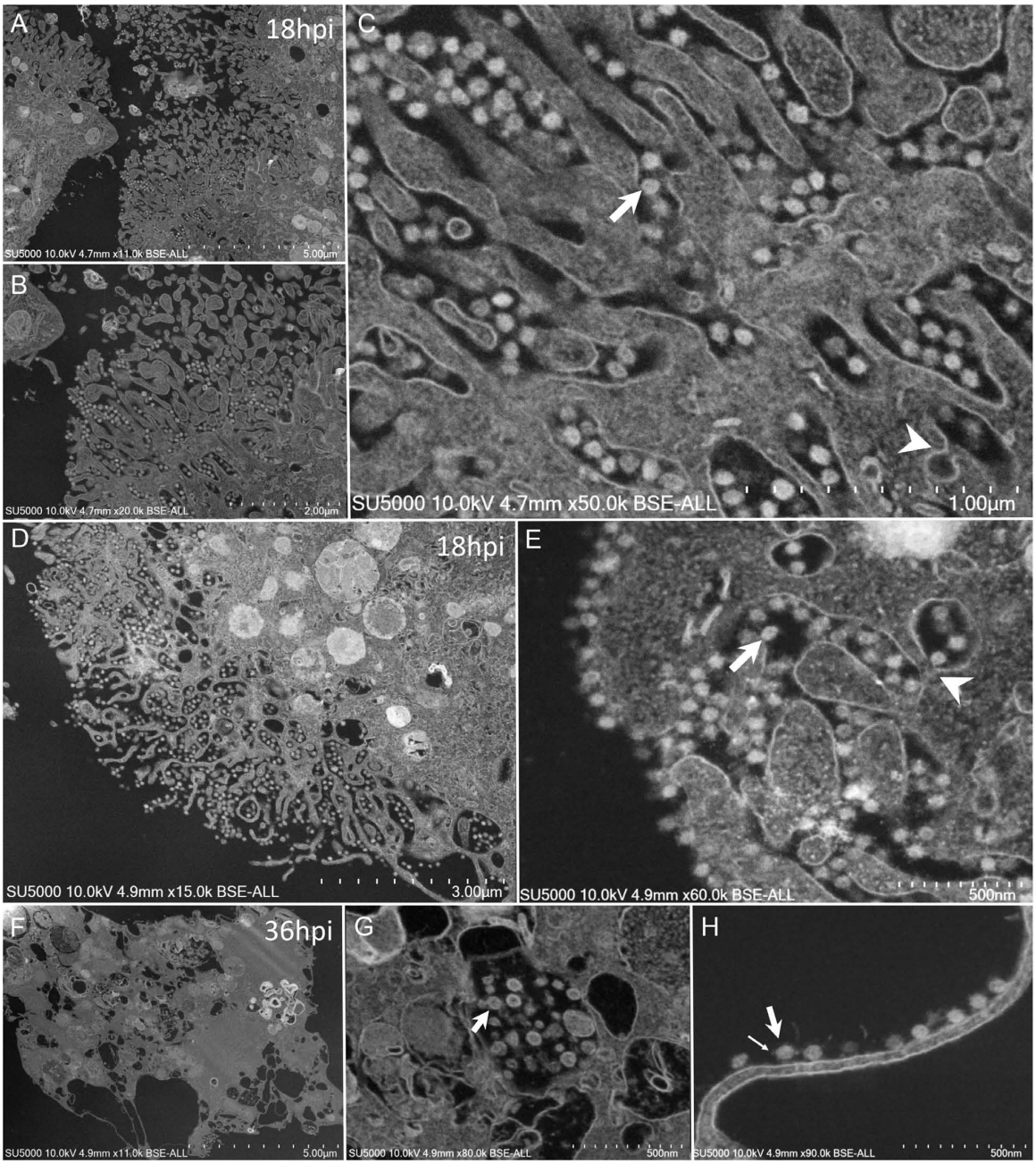


FIGURE 7 | SEM of SARS-CoV-2 mature particles at 18–36 h post-infection. observed at intra- and extra- cellular locations. **(A–C)** Viral mature particles lying between cellular microvilli. **(D,E)** Virus particles within smooth vesicles at the cell periphery and eventually fused with the cell membrane (arrowhead) to release the virus (arrow). **(F,G)** Viral particles in translucent vacuoles or attached to the plasma membrane of a lysed cell. Small arrow points to a SARS-CoV-2 corona spike.

electron-dense structures (also observed in SARS-CoV infectious cycle studies) probably correspond to assemblies of virus genomes together with helical nucleocapsids (Ng et al., 2003). As in SARS-CoV-2 infected cells, myelin-like membrane whorls have been previously described in SARS-CoV infected cells, closely associated with nascent particles (Ng et al., 2003). Although these membrane whorls were also present in uninfected cells, we hypothesize that these compartments may be derived from the ER and/or may be part of an auto-phago-(lyso) somal process, both scenarios providing a support for virions packaging and trafficking until further extracellular release. The electron-density and homogeneity difference of the VMMV containing viral particles may be related to the pH of these compartments and/or to the maturation level of the virions. The circular aspect of SARS-CoV-2 assemblies inside some VMMVs may reflect the presence of mature particles, compared to immature and

dispersed particles in less organized VMMV. As for SARS-CoV infected cells, one of the most obvious ultrastructural changes in SARS-CoV-2 infected cells was the proliferation of the Golgi complexes and related vesicles, accompanied by swelling of some of the Golgi sacs. We also found that VMMVs are most probably derived from the ER. It was shown that SARS-CoV nucleocapsids assemble in the ER and mature by budding into smooth vesicles derived from the GA. In parallel, the GA swells to form smooth vesicles that incorporate the VMMV along with their nucleocapsids (Oshiro et al., 1971; Patterson and Macnaughton, 1982; Zhang et al., 2003; Qinfen et al., 2004; Siu et al., 2008). Rather, our images suggest that nascent particles may bud in the cytoplasm, followed or simultaneous to the filling of the ER-derived VMMV by immature Golgi-derived virions. Nevertheless, the involvement of the Endoplasmic Reticulum – Golgi Apparatus complex witnessed here, especially the extreme budding of the Golgi apparatus, into the morphogenesis of the SARS-CoV-2, is consistent with what was demonstrated in chloroquine's efficacy against SARS-CoV-2 *in vitro* (Andreani et al., 2020; Wang et al., 2020). Indeed, chloroquine is a weak base which interferes with cell trafficking by increasing the pH of intracellular compartments (Devaux et al., 2020), especially lysosomes, and was shown to severely affects the endo-lysosomal system and the Golgi complex *in vitro* and *in vivo* (Mauthe et al., 2018).

Regarding SARS-CoV-2 cell exit, mature virions exited the cells at their apical sides, as observed for SARS-CoV (Rossen et al., 1994). The release of mature particles occurred passively in lysed cells or by fusion of the internal compartments with the plasma membrane in intact cells, as previously described (Oshiro et al., 1971; Ng et al., 2003; Qinfen et al., 2004).

Scanning electron microscopy (SEM) has shown here two main advantages over transmission electron microscopy (TEM) for studying SARS-CoV-2 life cycle. The first advantage came from the possibility to load at one time in the microscope several grids with ultra-thin sections of resin-embedded infected cells, corresponding to different post-infection times. All post infection times were thus accessible within 10 min in the SEM. The second advantage was that after SEM electron beam alignment and focus were adjusted, SEM screening of the ultra-thin sections was fast, compared to TEM, as only minor adjustments of the focus distance were needed when zooming on cells of interest or when moving from one grid to another. For TEM, focus adjustment was generally required for each position and magnification, and thus more time consuming. The whole SARS-CoV-2 infectious cycle was thus accessible at once, in a few hours of observation (4.5 h for 8 time-points, acquiring 320 micrographs), with possibilities to image at the cell population, cellular and sub-cellular levels. A similar screening of the same grids by TEM was more time consuming (around 8 h).

CONCLUSION

In conclusion, SEM has proven to be a rapid and effective tool for studying the SARS-CoV-2 infectious cycle in Vero cells. Further studies employing the same straightforward methodology may help understand at the cellular level the impact of pharmacological reagents on SARS-CoV-2 life cycle to better control this global pandemic.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

DB, J-PB, GH, ML, AF, and JB did the experiments and analyzed the data. DB, J-PB, and GH wrote the manuscript. J-PB, JB, DR, and BL conceived the project, supervised the experiments, and wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the French Government under the "Investments for the Future" program managed by the National Agency for Research (ANR), Méditerranée-Infection 10-IAHU-03 and was also supported by Région Provence-Alpes-Côte d'Azur and European funding FEDER PRIMMI (Fonds Européen de Développement Régional – Plateformes de Recherche et d'Innovation Mutualisées Méditerranée Infection). In addition, collaborative study conducted by IHU Méditerranée Infection and the Hitachi High-Tech Corporation was funded by the Hitachi High Tech Corporation. Funding sources had no role in the design and conduct of the study, the collection, management, analysis and interpretation of the data, nor in the preparation, review or approval of the manuscript.

ACKNOWLEDGMENTS

We sincerely thank Rita Jaafar, Isabelle Duflot, and Julien Andreani for their technical help. We also thank Takashi Irie, Kyoko Imai, Shigeki Matsubara, Taku Sakazume, Toshihide Agemura, Yusuke Ominami, and the Hitachi team in Japan from Hitachi High-Tech Corporation and Hitachi Ltd. for the collaborative study conducted together with IHU Méditerranée Infection, and for the installation of a SU5000 microscope at the IHU Méditerranée Infection facility.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.02014/full#supplementary-material

FIGURE S1 | SEM of uninfected Vero E6 cell showing electron-dense crescent-shaped in the cytoplasm (solid arrowheads), and a myelin-like membranes whorl (mw) in the perinuclear region.

FIGURE S2 | SARS-CoV-2 infected Vero E6 cells at 12–24 h post-infection. **(A–H)** SEM **(A,G,H)** and TEM **(B–F)** images showing numerous round and empty objects (arrows) at nucleus margins (n.m). **(A–E,G)** Images of nuclear membrane budding sites observed in transverse views. **(F,H)** Images of nuclear membrane budding sites in tangential views. SARS-CoV-2 particles can be seen outside the cells at cell plasma membranes [solid arrows in **(A,B)**, which correspond to the same cellular region].

FIGURE S3 | SEM of uninfected Vero E6 cell image showing round and empty objects (arrows) at nucleus margins **(A,B)** (arrows).

REFERENCES

Almeida, J. D., and Tyrrell, D. A. (1967). The morphology of three previously uncharacterized human respiratory viruses that grow in organ culture. *J. Gen. Virol.* 1, 175–178. doi: 10.1099/0022-1317-1-2-175

Alsaad, K. O., Hajeer, A. H., Al Balwi, M., Al Moaiqel, M., Al Oudah, N., Al Ajlan, A., et al. (2018). Histopathology of Middle East respiratory syndrome coronovirus (MERS-CoV) infection - clinicopathological and ultrastructural study. *Histopathology* 72, 516–524. doi: 10.1111/his.13379

Andreani, J., Le Bideau, M., Duflot, I., Jardot, P., Rolland, C., Boxberger, M., et al. (2020). In vitro testing of combined hydroxychloroquine and azithromycin on SARS-CoV-2 shows synergistic effect. *Microb. Pathog.* 145:104228. doi: 10.1016/j.micpath.2020.104228

Bardina, M. V., Lidsky, P. V., Sheval, E. V., Fominykh, K. V., van Kuppeveld, F. J. M., Polyakov, V. Y., et al. (2009). Mengovirus-induced rearrangement of the nuclear pore complex: hijacking cellular phosphorylation machinery. *J. Virol.* 83, 3150–3161. doi: 10.1128/JVI.01456-08

Caly, L., Druce, J., Roberts, J., Bond, K., Tran, T., Kostecki, R., et al. (2020). Isolation and rapid sharing of the 2019 novel coronavirus (SARS-CoV-2) from the first patient diagnosed with COVID-19 in Australia. *Med. J. Aust.* 212, 459–462. doi: 10.5694/mja2.50569

Colson, P., Lagier, J.-C., Baudoin, J.-P., Bou Khalil, J., La Scola, B., and Raoult, D. (2020). Ultrarapid diagnosis, microscope imaging, genome sequencing, and culture isolation of SARS-CoV-2. *Eur. J. Clin. Microbiol. Infect. Dis.* doi: 10.1007/s10096-020-03869-w [Epub ahead of print].

Curry, A., Appleton, H., and Dowsett, B. (2006). Application of transmission electron microscopy to the clinical study of viral and bacterial infections: present and future. *Micron* 37, 91–106. doi: 10.1016/j.micron.2005.10.001

de Castro, I. F., Volonté, L., and Risco, C. (2013). Virus factories: biogenesis and structural design. *Cell. Microbiol.* 15, 24–34. doi: 10.1111/cmi.1202

Devaux, C. A., Rolain, J.-M., Colson, P., and Raoult, D. (2020). New insights on the antiviral effects of chloroquine against coronavirus: what to expect for COVID-19? *Int. J. Antimicrob. Agents* 55:105938. doi: 10.1016/j.ijantimicag.2020.105938

Golding, C. G., Lamboo, L. L., Beniac, D. R., and Booth, T. F. (2016). The scanning electron microscope in microbiology and diagnosis of infectious disease. *Sci. Rep.* 6:26516. doi: 10.1038/srep26516

Goldsmith, C. S., and Miller, S. E. (2009). Modern uses of electron microscopy for detection of viruses. *Clin. Microbiol. Rev.* 22, 552–563. doi: 10.1128/CMR.00027-09

Hazelton, P. R., and Gelderblom, H. R. (2003). Electron microscopy for rapid diagnosis of infectious agents in emergent situations. *Emerg. Infect. Dis.* 9, 294–303. doi: 10.3201/eid0903.020327

Kim, J.-M., Chung, Y.-S., Jo, H. J., Lee, N.-J., Kim, M. S., Woo, S. H., et al. (2020). Identification of Coronavirus isolated from a patient in Korea with COVID-19. *Osong. Public Health Res. Perspect.* 11, 3–7. doi: 10.24171/j.phrp.2020.11.1.02

Kim, S.-H., Chang, S. Y., Sung, M., Park, J. H., Bin Kim, H., Lee, H., et al. (2016). Extensive viable middle east respiratory syndrome (MERS) Coronavirus contamination in air and surrounding environment in MERS isolation wards. *Clin. Infect. Dis.* 63, 363–369.

Koster, A. J., and Klumperman, J. (2003). Electron microscopy in cell biology: integrating structure and function. *Nat. Rev. Mol. Cell Biol.* Suppl, SS6–SS10. doi: 10.1038/nrm1194

Lin, D. H., and Hoelz, A. (2019). The structure of the nuclear pore complex (an update). *Annu. Rev. Biochem.* 88, 725–783. doi: 10.1146/annurev-biochem-062917-011901

Mauthe, M., Orhon, I., Rocchi, C., Zhou, X., Luhr, M., Hijlkema, K.-J., et al. (2018). Chloroquine inhibits autophagic flux by decreasing autophagosome-lysosome fusion. *Autophagy* 14, 1435–1455. doi: 10.1080/15548627.2018.1474314

Ng, M. L., Tan, S. H., See, E. E., Ooi, E. E., and Ling, A. E. (2003). Early events of SARS coronavirus infection in vero cells. *J. Med. Virol.* 71, 323–331. doi: 10.1002/jmv.10499

Oshiro, L. S., Schieble, J. H., and Lennette, E. H. (1971). Electron microscopic studies of Coronavirus. *J. Gen. Virol.* 12, 161–168. doi: 10.1099/0022-1317-12-2-161

Park, W. B., Kwon, N.-J., Choe, P. G., Choi, S.-J., Oh, H. S., Lee, S. M., et al. (2016). Isolation of middle east respiratory syndrome Coronavirus from a patient of the 2015 Korean outbreak. *J. Korean Med. Sci.* 31, 315–320. doi: 10.3346/jkms.2016.31.2.315

Patterson, S., and Macnaughton, M. R. (1982). Replication of human respiratory Coronavirus strain 229E in human macrophages. *J. Gen. Virol.* 60, 307–314. doi: 10.1099/0022-1317-60-2-307

Qinfen, Z., Jinming, C., Xiaojun, H., Huanying, Z., Jicheng, H., Ling, F., et al. (2004). The life cycle of SARS coronavirus in Vero E6 cells. *J. Med. Virol.* 73, 332–337. doi: 10.1002/jmv.20095

Reynolds, E. S. (1963). The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell Biol.* 17, 208–212. doi: 10.1083/jcb.17.1.208

Rossen, J. W., Bekker, C. P., Voorhout, W. F., Strous, G. J., van der Ende, A., and Rottier, P. J. (1994). Entry and release of transmissible gastroenteritis coronavirus are restricted to apical surfaces of polarized epithelial cells. *J. Virol.* 68, 7966–7973. doi: 10.1128/jvi.68.12.7966-7973.1994

Schoeman, D., and Fielding, B. C. (2019). Coronavirus envelope protein: current knowledge. *Virol. J.* 16:69. doi: 10.1186/s12985-019-1182-0

Siu, Y. L., Teoh, K. T., Lo, J., Chan, C. M., Kien, F., Escriou, N., et al. (2008). The M, E, and N structural proteins of the severe acute respiratory syndrome coronavirus are required for efficient assembly, trafficking, and release of virus-like particles. *J. Virol.* 82, 11318–11330. doi: 10.1128/JVI.01052-08

Wang, M., Cao, R., Zhang, L., Yang, X., Liu, J., Xu, M., et al. (2020). Remdesivir and chloroquine effectively inhibit the recently emerged novel coronavirus (2019-nCoV) in vitro. *Cell Res.* 30, 269–271. doi: 10.1038/s41422-020-0282-0

Yuan, X., Yao, Z., Shan, Y., Chen, B., Yang, Z., Wu, J., et al. (2005). Nucleolar localization of non-structural protein 3b, a protein specifically encoded by the severe acute respiratory syndrome coronavirus. *Virus Res.* 114, 70–79. doi: 10.1016/j.virusres.2005.06.001

Zhang, Q.-F., Cui, J.-M., Huang, X.-J., Lin, W., Tan, D.-Y., Xu, J.-W., et al. (2003). Morphology and morphogenesis of severe acute respiratory syndrome (SARS)-associated virus. *Sheng Wu Hua Xue Yu Sheng Wu Wu Li Xue Bao* 35, 587–591.

Zhu, N., Zhang, D., Wang, W., Li, X., Yang, B., Song, J., et al. (2020). A novel Coronavirus from patients with pneumonia in China, 2019. *New Engl. J. Med.* 382, 727–733. doi: 10.1056/NEJMoa2001017

Conflict of Interest: DR was a consultant for the Hitachi High-Tech Corporation.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Medicine

ORIGINAL RESEARCH
published: 19 August 2020
doi: 10.3389/fmed.2020.00530

Exploring the Demographics and Clinical Characteristics Related to the Expression of Angiotensin-Converting Enzyme 2, a Receptor of SARS-CoV-2

Shengjie Li, Jianping Han, Aiping Zhang, Yi Han, Miaomiao Chen, Zhenzhen Liu, Mingxi Shao and Wenjun Cao**

Clinical Laboratory, Eye & ENT Hospital, Fudan University, Shanghai, China

OPEN ACCESS

Edited by:
Ata Murat Kaynar, University of Pittsburgh, United States

Reviewed by:
Pedro Xavier-Elsas, Federal University of Rio de Janeiro, Brazil
Guangqiang Wang, University of Shanghai for Science and Technology, China

***Correspondence:**
Shengjie Li
lishengjie6363020@163.com
Wenjun Cao
wgkjyk@aliyun.com

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: *03 April 2020*
Accepted: *27 July 2020*
Published: *19 August 2020*

Citation:
Li S, Han J, Zhang A, Han Y, Chen M, Liu Z, Shao M and Cao W (2020) Exploring the Demographics and Clinical Characteristics Related to the Expression of Angiotensin-Converting Enzyme 2, a Receptor of SARS-CoV-2. Front. Med. 7:530. doi: 10.3389/fmed.2020.00530

Objective: Coronavirus disease 2019 (COVID-19) was first reported in Wuhan, China, and has rapidly spread throughout the world. It has been reported that angiotensin-converting enzyme 2 (ACE2) is one of the major cellular entry receptors of SARS-CoV-2; thus, high ACE2 expression may increase susceptibility to infection. Therefore, we analyzed the expression of ACE2 in the blood to identify the individuals who may be susceptible to infection.

Methods: In total, 229 subjects were enrolled in this study, and reverse transcription-quantitative polymerase chain reaction and ELISA assay was used to identify the level of ACE2 mRNA expression and ACE2 protein level in the blood. Demographic and clinical characteristics, including age, gender, weight, height, smoking habits, drinking habits, diabetes, and hypertension, were obtained using a face-to-face questionnaire. Independent Student's *t*-test, Pearson's linear correlation, logistic regression analysis, and multiple linear regression correlation were performed to assess the association between these factors and the expression of ACE2.

Results: Higher level of ACE2 was observed in females, older subjects, subjects with hypertension, subjects with a cardiocerebrovascular disease, male smokers, and subjects with cancer ($p < 0.05$) than in other subjects. Multiple linear regression analysis showed that there is a statistically significant correlation between being a female and ACE2 expression ($\beta = 0.550$, $p < 0.001$), between older age and ACE2 expression ($\beta = 0.197$, $p = 0.003$), between smoking and ACE2 expression ($\beta = 0.163$, $p = 0.037$), and between cancer and ACE2 expression ($\beta = 0.265$, $p < 0.001$). Logistic regression analysis revealed that female subjects (odds ratio [OR] = 2.255, 95% confidence interval [CI] = 1.770–2.872), subjects with hypertension (OR = 1.264, 95% CI = 1.075–1.486), subjects with a cardiocerebrovascular disease (OR = 1.271, 95% CI = 1.023–1.579), subjects with cancer (OR = 1.695, 95% CI = 1.253–2.293), and subjects above 60 years of age (OR = 3.097, 95% CI = 1.078–8.896) are at an increased risk of infection due to their high expression of ACE2.

Conclusion: The level of ACE2 is higher in females, older subjects, smokers, and subjects with cancer than in other subjects, indicating that some of which are at higher risk for the severe forms of COVID-19 when they are exposed to the SARS-Cov-2.

Keywords: coronavirus disease 2019, angiotensin covering enzyme II, susceptibility, gender, age, smoking, cancer

INTRODUCTION

In December 2019, coronavirus disease 2019 (COVID-19) was detected in patients in Wuhan, Hubei Province, China (1). The virus started spreading rapidly throughout China and the world (2, 3). According to the World Health Organization, as of June 10, 2020, 7,145,539 laboratory-confirmed cases were detected, with a death toll of 408,025 patients (4). Given the rapid spreading of this outbreak, it is urgent to identify subjects who may be susceptible to infection and to further control the spread of the disease to those susceptible subjects.

It has been shown from severe acute respiratory syndrome coronavirus (SARS-Cov) and Middle East respiratory syndrome coronavirus (MERS-CoV) that humans exhibit disparities in susceptibility to these viruses (5–7). For example, Liu et al. (5) reported that older age (odds ratio [OR] = 8.546, 95% confidence interval [CI] = 1.628–44.864, $p = 0.011$) and smoking (OR = 14.285, 95% CI = 1.577–25.000, $p = 0.018$) are risk factors for the progression of COVID-19. Rao et al. (8) showed that over 25% of patients with COVID-19 have a history of hypertension (12.9%) and diabetes (5.4%). However, it is still unclear whether the above factors, or perhaps even other factors, are associated with susceptibility to COVID-19. It has been reported that angiotensin-converting enzyme 2 (ACE2) is one of the major cellular entry receptors of COVID-19 (9), indicating that a higher expression of ACE2 may lead to increased susceptibility to infection. Several studies have investigated the relationship between the expression level of ACE2 and the demographic or clinical characteristics of COVID-19. For example, Rao et al. performed a phenome-wide Mendelian randomization study and found that type II diabetes is causally linked to an increased expression of ACE2 (8). Chen et al. (10) showed that ACE2 is mainly expressed in the epithelial cells of the colon and that its expression is increased the most in patients with colorectal cancer followed by patients with adenoma, compared to healthy controls. Moreover, Cai (11) reported that ACE2 expression is significantly higher in the lungs of former smokers than in non-smokers. According to previous evidence, the expression level of ACE2 is associated with susceptibility to COVID-19 infection.

However, previous studies had several limitations. On the one hand, most of the samples were derived from different types of tissues, such as lung and colon tissues, which may not be fully reflective of the expression in the whole body. Another potential limitation was that the sample size was too small to draw conclusions. Therefore, in this study, we performed a cross-sectional study to explore the clinical/demographic characteristics that may lead to an increased expression of ACE2, which may in turn result in greater susceptibility to infection with COVID-19 when they are exposed to the SARS-Cov-2.

TABLE 1 | Demographics and clinical characteristics.

	Number of subjects/Mean value
Gender, male/female	125/104
Age, years	
Mean ± SD, male/female	51.94 ± 17.59/51.22 ± 16.96
0–20, male/female	9, 5/4
20–40, male/female	54, 29/25
40–60, male/female	76, 44/32
>60, male/female	90, 47/43
BMI, mean, Kg/m^2	
Mean ± SD, male	24.55 ± 3.77
Mean ± SD, female	22.61 ± 3.67
Smoking habit, proportion% (yes)	
Male	37.60 (47)
Female	4.81 (5)
Drinking habit, proportion% (yes)	
Male	20.80 (26)
Female	2.88 (3)
Diabetes mellitus, proportion% (yes)	
Male	15.20 (19)
Female	11.54 (12)
Hypertension, proportion% (yes)	
Male	26.40 (33)
Female	36.54 (38)
Cardio-cerebrovascular disease, proportion% (yes)	
Male	9.60 (12)
Female	11.54 (12)
Cancer, proportion% (yes)	
Male	4.00 (5)
Female	5.77 (6)

MATERIALS AND METHODS

Subjects

This study was conducted at the Department of clinical laboratory, Eye, Ear, Nose and Throat (Eye and ENT) Hospital of Fudan University, Shanghai, China, and was approved by the Ethics Committee of the same hospital. This study adhered to the principles of the Declaration of Helsinki. Informed consent was obtained from all subjects. All subjects were recruited from the Eye and ENT Hospital of Fudan University.

Examination

Medical examinations, including the assessment of electrocardiograms, X-rays, liver function, blood glucose, infectious diseases, renal function, blood pressure, heart rate,

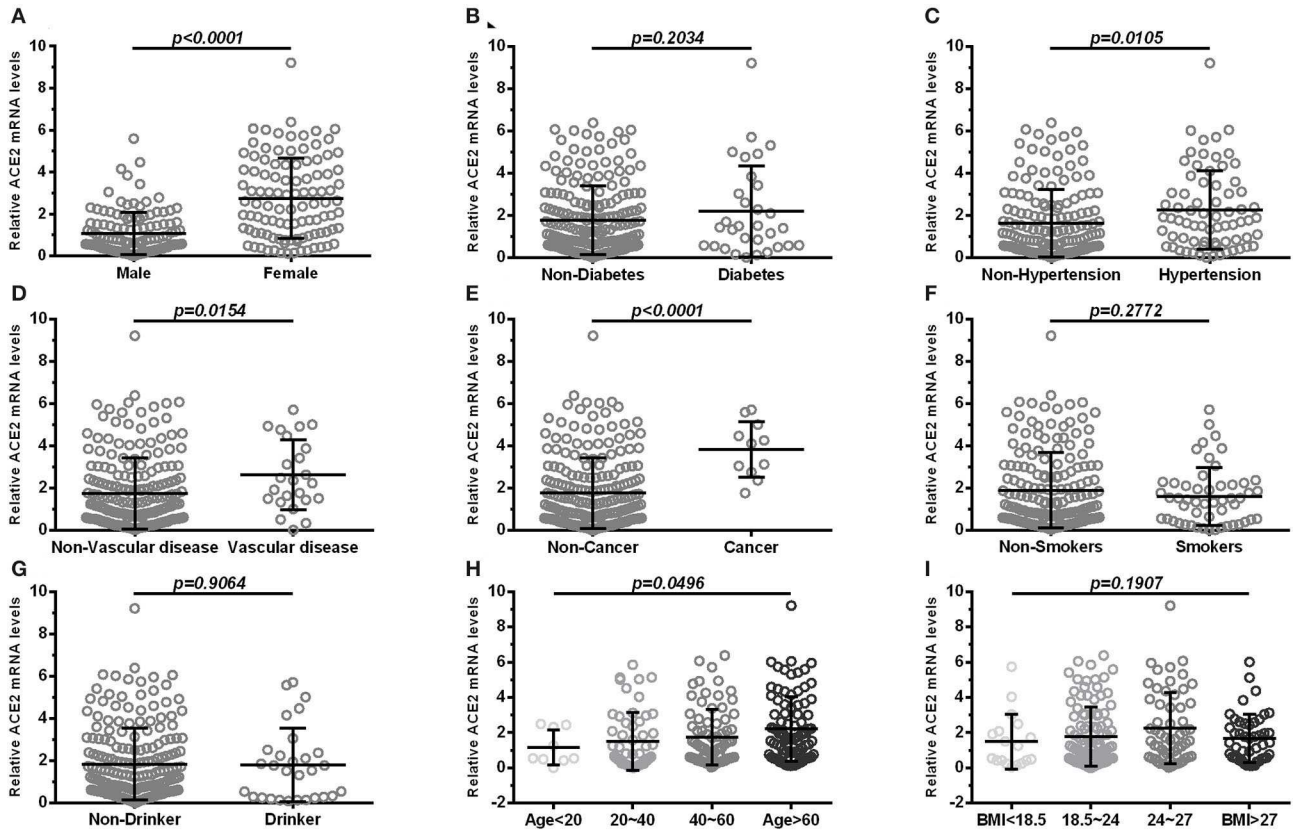


FIGURE 1 | Comparison of ACE2 mRNA levels in subjects with different demographic and clinical characteristics **(A)** shows expression in blood of male and female; **(B)** shows expression in blood of diabetes and non-diabetes; **(C)** shows expression in blood of hypertension and non-hypertension; **(D)** shows expression in blood of vascular disease and non-vascular disease; **(E)** shows expression in blood of cancer and non-cancer; **(F)** shows expression in blood of smokers and non-smokers; **(G)** shows expression in blood of drinker and non-drinker; **(H,I)** shows groups in age (<20, 20–40, 40–60, >60) and BMI (<18.5, 18.5–24, 24–27, >27). Vascular, cardiocerebrovascular disease; BMI, body mass index. Each data point represents one subject. Data are expressed as mean ± SD. Independent Student's *t*-test and Mann–Whitney *U*-test were used.

body temperature, height, and weight, were performed for all subjects by the respective specialty physicians at the Eye and ENT Hospital of Fudan University. Demographic and clinical characteristics, including age, gender, weight, height, smoking habits, drinking habits, diabetes, and hypertension, were collected using a face-to-face questionnaire. Body mass index (BMI) was calculated as the weight in kilograms divided by the height in meters squared. Drinking was defined as more than three drinks per week for more than 6 months (current or former), and smoking was defined as more than one cigarette per day for more than 6 months (current or former) (12).

The exclusion criteria were as follows: patients with an autoimmune disease, patients with an acute infectious disease, patients with a metabolic syndrome, patients who have undergone surgery within the previous 2 months, patients with abnormal hepatic or renal function, patients with a hereditary disease, and patients whose body temperature was above 37.5°C. Hence, a total of 20 subjects (surgery = 5, metabolic syndrome = 5, autoimmune disease = 4, acute infectious disease = 4, and hereditary disease = 2) were excluded.

RNA Isolation and Detection

Blood samples were obtained in the morning after subjects had fasted for 8 h via standard venipuncture in the antecubital fossae (anterior elbow veins). First, blood samples (2 mL) were collected in ethylenediaminetetraacetic acid (EDTA) tubes. Total RNA was extracted using a TRIzol reagent (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) as per the manufacturer's instructions. The quality and integrity of the acquired total RNA were evaluated using a NanoDrop™ 2000c (Thermo Fisher Scientific, Inc., Wilmington, DE, USA). For reverse transcription-quantitative polymerase chain reaction (RT-qPCR), 1,000 ng of total RNA was reverse-transcribed with 2 μL of 5X OneStep RT Mix. The RT-qPCR reaction was performed using 1 μL of RT products, 0.2 μL of 10 μM forward primer, 0.2 μL of 10 μM pmol reverse primer, and 5 μL of 2X SYBR Green I qPCR mix and completed to 10 μL with nuclease-free water. The primers used were as follows: ACE2-forward, 5′-AAAGGAACAGTCCACACTTGCCC-3′, and ACE2-reverse, 5′-TGAAGACCCATTTTGCTGAAGAGCC-3′.

TABLE 2 | Comparison of ACE2 protein levels in subjects with different demographic and clinical characteristics.

	ACE2	*t*-value	*P*-value
Gender			
Male	16.28 ± 5.87		
Female	2,009 ± 5.63	4.983	<0.001
Diabetes			
Yes	19.63 ± 6.15		
No	17.75 ± 6.02	1.609	0.109
Hypertension			
Yes	20.24 ± 5.54		
No	17.01 ± 6.02	3.956	<0.001
Vascular disease			
Yes	21.97 ± 5.58		
No	17.53 ± 5.94	3.477	0.001
Cancer			
Yes	27.52 ± 2.44		
No	17.52 ± 5.78	11.977	<0.001
Smoking			
Yes	19.65 ± 5.77		
No	17.52 ± 6.07	2.318	0.023
Drinking			
Yes	18.81 ± 7.85		
No	17.88 ± 5.76	0.612	0.545
Age, years			
<20	15.67 ± 6.31		
20–40	15.94 ± 7.09		
40–60	18.51 ± 5.58		
>60	19.05 ± 5.44	3.733	0.012
BMI, Kg/M^2			
<18.5	16.69 ± 5.88		
18.5–24	17.98 ± 6.14		
24–27	18.34 ± 6.16		
>27	18.08 ± 6.07	0.323	0.809

ELISA Assay

The protein level of ACE2 was further measured by ELISA kit. The serum samples were subjected to ACE2 assay as described in the ACE2 assay kit (ab235649, Abcam, USA). The ACE2 concentration of each sample was detected by multimode microplate readers (Biotek SynergyH1, USA) at 450 nm.

Statistical Analysis

All analyses were performed using the Statistical Package for the Social Sciences software, version 13.0 (SPSS Inc., Chicago, IL, USA). Figures were created using GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA). Results are presented as mean ± standard deviation (SD). Normality was assessed using the Kolmogorov–Smirnoff test. An independent Student's *t*-test, Mann-Whitney *U*-test, Pearson's analysis, and one-way analysis of variance (ANOVA) were used. Multivariate linear regression analysis was performed to evaluate the association between ACE2 levels and factors. Logistic regression analysis was performed to estimate the ORs with 95% CIs. A $p < 0.05$ was considered statistically significant.

RESULTS

Characteristics of the Study Patients

A total of 229 subjects (125 males, 104 females) were enrolled, which was conducted at the Eye and ENT Hospital of Fudan University. The mean age of the males and females was 51.94 ± 17.59 years and 51.22 ± 16.96 years, respectively. Among all subjects, the proportion of smoking history (37.6 vs. 4.81%) and drinking history (20.8 vs. 2.88%) was significantly higher in male subjects than in female subjects. Moreover, the prevalence of diabetes, hypertension, cardiocerebrovascular diseases, and cancer was similar between male and female subjects. The demographic and clinical characteristics of the subjects are shown in **Table 1**.

Comparison of ACE2 mRNA and Protein Levels in Subjects With Different Demographic and Clinical Characteristics

ACE2 expression was higher in female subjects than in male subjects ($p < 0.0001$; **Figure 1A**). There was no significant differences ($p > 0.05$) in the expression of ACE2 between subjects with diabetes and subjects without (**Figure 1B**). Furthermore, subjects with hypertension (**Figure 1C**), cardiocerebrovascular diseases (**Figure 1D**), and cancer (**Figure 1E**) exhibited a higher ACE2 expression than that of those not suffering from these diseases (both $p < 0.05$). Moreover, we observed no significant differences ($p > 0.05$) in the expression of ACE2 between smokers and non-smokers (**Figure 1F**), and between drinkers and non-drinkers (**Figure 1G**). The expression of ACE2 was lowest in the >60 age group followed by the 40–60, 20–40, and <20 age groups, and the differences were statistically significant ($p = 0.0496$; **Figure 1H**). There was no significant differences ($p > 0.05$) in the expression of ACE2 among BMI subgroup (**Figure 1I**). A similar result was observed when ACE2 protein levels were compared in subjects with different demographic and clinical characteristics (**Table 2**).

Comparison of ACE2 mRNA and Protein Levels in Subjects With Different Demographic and Clinical Characteristics, Stratified According to Sex

According to sex, all subjects were divided into male and female subgroups. The expression of ACE2 was found to be lowest in the >60 age group followed by the 40–60, 20–40, and <20 age groups in both males (**Figure 2A**) and females (**Figure 2B**). Moreover, we observed no significant differences ($p > 0.05$) in the expression of ACE2 among BMI subgroup (**Figures 2C,D**), and between subjects with diabetes and subjects without (**Figures 2E,F**) in the male and female subgroup. In both male and female subgroups, a higher ACE2 expression was observed in subjects with hypertension (**Figures 2G,H**), cardiocerebrovascular diseases

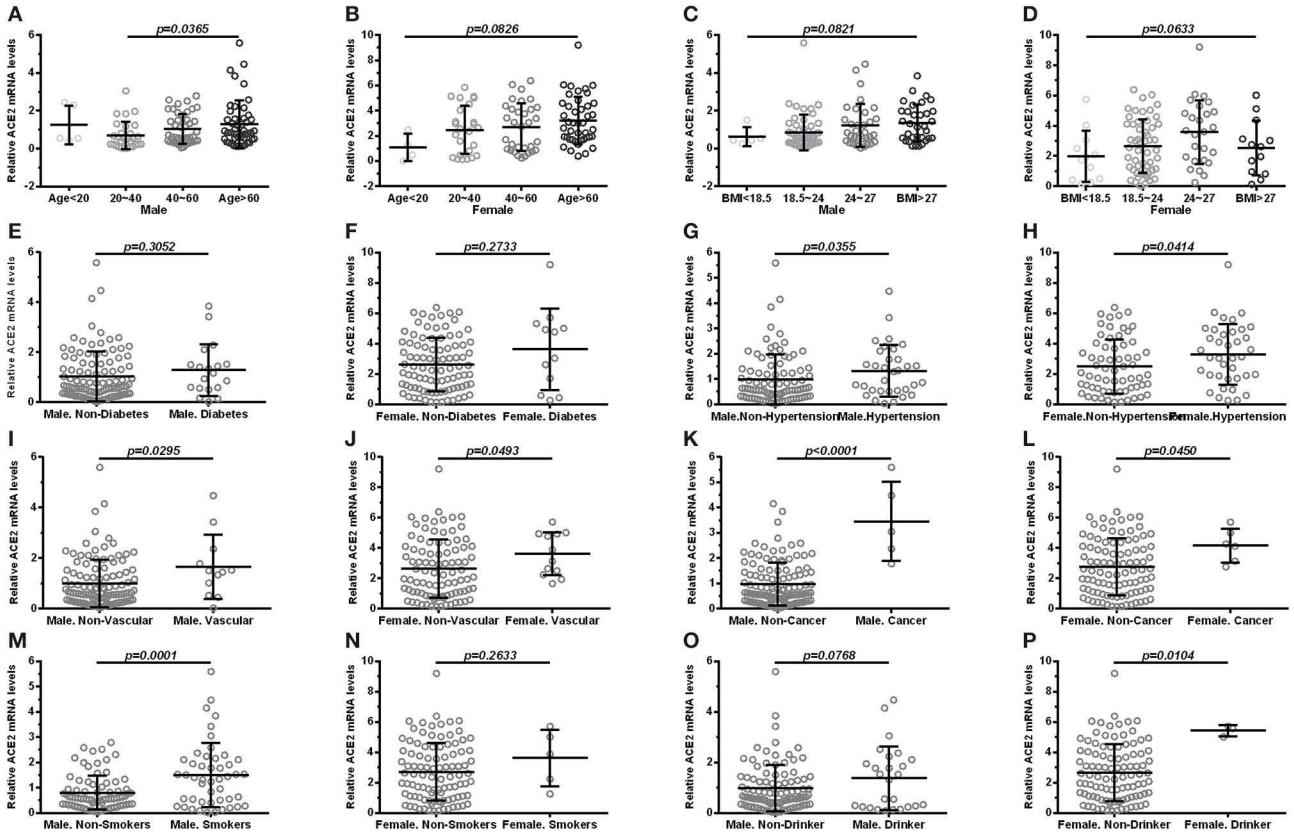


FIGURE 2 | Comparison of ACE2 mRNA levels in subjects with different demographic and clinical characteristics, stratified according to sex. (male: **A**, **C**, **E**, **G**, **I**, **K**, **M**, **O**; female: **B**, **D**, **F**, **H**, **J**, **L**, **N**, **P**). **(A,B)** shows groups in age (<20, 20–40, 40–60, >60); **(C,D)** shows groups in BMI (<18.5, 18.5–24, 24–27, >27); **(E,F)** shows expression in diabetes and non-diabetes; **(G,H)** shows expression in hypertension and non-hypertension; **(I,J)** shows expression in vascular disease and non-vascular disease; **(K,L)** shows expression in cancer and non-cancer; **(M,N)** shows expression in smokers and non-smokers; **(O,P)** shows expression in drinker and non-drinker. Vascular, cardiocerebrovascular disease; BMI, body mass index. Each data point represents one subject. Data are expressed as mean ± SD. One-way ANOVA and Mann–Whitney *U*-test were used.

(**Figures 2I,J**), and cancer (**Figures 2K,L**), with $p < 0.05$. Male smokers exhibited a significantly higher ACE2 expression ($p = 0.0001$; **Figure 2M**) compared to male non-smokers, but not in females ($p = 0.2633$; **Figure 2N**). Interestingly, female drinkers exhibited a higher ACE2 expression compared to non-drinkers ($p = 0.0104$; **Figure 2P**), but not in males (**Figure 2O**). A similar result was observed when ACE2 protein levels were compared in subjects with different demographic and clinical characteristics, stratified according to sex (**Table 3**).

Pearson's analysis also showed that there was a significantly positive correlation between age and the expression level of ACE2 (**Figure 3A**), in both males (**Figure 3B**) and females (**Figure 3C**). Although we observed no significant association between BMI and the expression level of ACE2 in any of the subjects (**Figure 3D**), a significant correlation was also found in both male (**Figure 3E**) and female subgroup (**Figure 3F**). Furthermore, there was a significantly positive correlation between ACE2 mRNA levels and ACE2 protein levels ($r = 0.677$, $p < 0.001$).

Multiple Linear Regression for the Association Between Age, Gender, Smoking Habits, and Cancer and ACE2 mRNA Levels

After adjusting for age, sex, BMI, hypertension, diabetes, drinking habits, smoking habits, cardiocerebrovascular diseases, and cancer, multiple linear regression analysis showed that there is a statistically significant correlation between being a female and ACE2 expression ($\beta = 0.550$, $p < 0.001$), between older age and ACE2 expression ($\beta = 0.197$, $p = 0.003$), between smoking and ACE2 expression ($\beta = 0.163$, $p = 0.037$), and between cancer and ACE2 expression ($\beta = 0.265$, $p < 0.001$). In the sex-stratified subgroup, adjusting for age, BMI, hypertension, diabetes, drinking habits, smoking habits, cardiocerebrovascular diseases, and cancer, similar results were also observed in the male and female subgroups. However, a relationship between smoking and ACE2 expression was just observed in the male subgroup, but not in the female subgroup (see **Table 4** for details).

TABLE 3 | Comparison of ACE2 protein levels in subjects with different demographic and clinical characteristics, stratified according to sex.

	Male	*P*-value	Female	*P*-value
Diabetes				
Yes	18.15 ± 5.12		21.95 ± 7.12	
No	15.94 ± 5.95	0.133	19.84 ± 5.40	0.224
Hypertension				
Yes	18.99 ± 5.27		21.59 ± 5.44	
No	15.31 ± 5.79	0.002	19.22 ± 5.57	0.035
Vascular disease				
Yes	21.21 ± 6.47		22.79 ± 4.50	
No	15.76 ± 5.58	0.002	19.75 ± 5.68	0.041
Cancer				
Yes	28.40 ± 2.67		26.78 ± 2.17	
No	15.78 ± 5.41	<0.001	19.73 ± 5.51	0.002
Smoking				
Yes	19.14 ± 5.72		24.43 ± 4.02	
No	14.56 ± 5.28	<0.001	19.91 ± 5.61	0.079
Drinking				
Yes	17.96 ± 7.77		26.18 ± 4.05	
No	15.84 ± 5.22	0.198	19.95 ± 5.57	0.109
Age, years				
<20	16.60 ± 4.87		14.49 ± 8.45	
20–40	14.14 ± 6.45		18.03 ± 7.35	
40–60	16.72 ± 5.61		20.32 ± 4.99	
>60	17.20 ± 5.67	0.030	21.73 ± 3.87	0.010
BMI, Kg/M^2				
<18.5	13.17 ± 1.76		18.16 ± 6.05	
18.5–24	15.33 ± 6.02		20.71 ± 5.02	
24–27	16.55 ± 5.48		20.61 ± 6.12	
>27	17.87 ± 6.14	0.159	18.61 ± 6.46	0.370

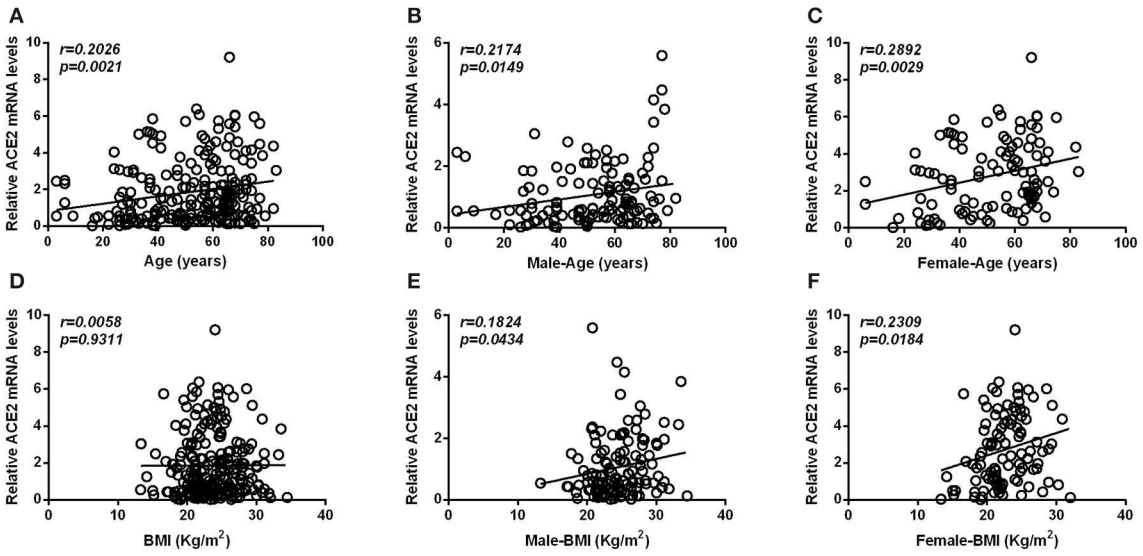


FIGURE 3 | Scatterplot of patient individual measurements for age and BMI vs. ACE2 mRNA levels. **(A–C)** Shows the association between ACE2 mRNA levels and age, between ACE2 mRNA levels and age in male subjects, and between ACE2 mRNA levels and age in female subjects. **(D–F)** Shows the association between ACE2 mRNA levels and BMI, between ACE2 mRNA levels and BMI in male subjects and between ACE2 mRNA levels and BMI in female subjects. Each data point represents one patient. Pearson's analysis was used.

TABLE 4 | Multiple linear correlation analysis to assess the relationship between age, gender, smoking, and cancer with ACE2 mRNA levels.

	Multiple linear correlation		Multiple linear correlation-Male		Multiple linear correlation-Female	
	Beta	***P* (95%CI)**	**Beta**	***P* (95%CI)**	**Beta**	***P* (95%CI)**
Female	0.550	<0.001 (1.473–2.310)	NA	NA	NA	NA
Older age	0.197	0.003 (0.007–0.032)	0.162	0.038 (0.001–0.018)	0.318	0.001 (0.015–0.057)
Smoking	0.163	0.037 (0.040–0.299)	0.184	0.043 (0.012–0.754)	NA	NA
Cancer	0.265	<0.001 (1.031–3.192)	0.477	<0.001 (1.650–3.203)	0.220	0.020 (0.283–3.296)

Beta, standardized coefficients.

TABLE 5 | Logistic regression analyses to identify risk factors for the ACE2 mRNA levels.

	Logistic regression		Logistic regression (Male)		Logistic regression (Female)	
	OR	**P (95%CI)**	**OR**	**P (95%CI)**	**OR**	**P (95%CI)**
Gender						
Male	1		NA	NA	NA	NA
Female	2.255	<0.001 (1.770–2.872)	NA	NA	NA	NA
Smoking						
No	1		1		1	
Yes	0.888	0.235 (0.730–1.080)	1.710	0.008 (1.149–2.544)	0.889	0.244 (0.729–1.084)
Drinking						
No	1		1		1	
Yes	0.977	0.847 (0.775–1.233)	1.414	0.086 (0.953–2.100)	0.967	0.776 (0.765–1.221)
Diabetes						
No	1		1		1	
Yes	1.182	0.105 (0.966–1.446)	1.256	0.307 (0.811–1.947)	1.180	0.112 (0.962–1.448)
Hypertension						
No	1		1		1	
Yes	1.264	0.005 (1.075–1.486)	1.845	0.003 (1.226–2.776)	1.254	0.006 (1.066–1.475)
Vascular						
No	1		1		1	
Yes	1.271	0.031 (1.023–1.579)	1.946	0.006 (1.208–3.137)	1.276	0.031 (1.023–1.591)
Cancer						
No	1		1		1	
Yes	1.695	0.001 (1.253–2.293)	4.720	0.001 (1.900–11.633)	1.685	0.001 (1.245–2.281)
Age						
<20	1		1		1	
20–40	1.175	0.539 (0.702–1.967)	2.802	0.378 (0.284–27.623)	1.758	0.186 (0.762–4.060)
40–60	2.365	0.094 (0.863–6.482)	10.370	0.144 (0.450–238.776)	2.086	0.153 (0.761–5.716)
>60	3.097	0.036 (1.078–8.896)	11.571	0.107 (0.592–226.323)	4.027	0.049 (1.003–16.176)
BMI						
<18.5	1		1		1	
18.5–24	1.074	0.667 (0.776–1.487)	1.46	0.611 (0.355–6.433)	1.278	0.222 (0.862–1.895)
24–27	1.245	0.204 (0.888–1.746)	2.427	0.282 (0.483–12.203)	1.532	0.067 (0.970–2.419)
>27	1.056	0.794 (0.702–1.588)	3.440	0.151 (0.636–18.596)	1.222	0.411 (0.785–1.972)

Vascular, cardiocerebrovascular disease; BMI, body mass index.

Logistic Regression Analysis to Identify the Risk Factors for the ACE2 mRNA Levels

After adjusting for age, sex, BMI, hypertension, diabetes, drinking habits, smoking habits, cardiocerebrovascular diseases, and cancer, logistic regression analysis revealed that females (OR = 2.255, 95% CI = 1.770–2.872), subjects with hypertension (OR = 1.264, 95% CI =1.075–1.486), subjects with cardiocerebrovascular diseases (OR = 1.271, 95% CI = 1.023–1.579), subjects with cancer (OR = 1.695, 95% CI = 1.253–2.293), and subjects above 60 years of age (OR = 3.097,

95% CI = 1.078–8.896) are at an increased risk for infection due to their high expression of ACE2. In both male and female subgroups, after adjusting for age, BMI, hypertension, diabetes, drinking habits, smoking habits, cardiocerebrovascular diseases, and cancer, similar results were also observed. Moreover, male smokers (OR = 1.710, 95% CI = 1.149–2.544) were found to be at an increased risk due to their high expression of ACE2, but not female subjects (see **Table 5** for details).

DISCUSSION

As far as we know, previous studies failed to comprehensively study many different risk factors/diseases and evaluate whether these are risk factors for COVID-19. In this study, we investigated the disparities related to age, gender, BMI, smoking habits, drinking habits, diabetes, hypertension, cardiocerebrovascular diseases, and cancer in ACE2 gene expression, which in turn may influence susceptibility to infection with COVID-19.

From our analysis, the most credible finding was the link between gender, age, smoking habits (male subjects), and cancer and the expression of ACE2, which was supported by an independent Student's *t*-test, multivariate linear regression analysis, and logistic regression analysis. Other results are preliminary but are worthy of further studies. For example, hypertension and cardiocerebrovascular diseases were found to cause a higher expression of ACE2, and showed positive associations with the expression of ACE2.

Numerous studies showed that older age is associated with susceptibility to infection and the presence of a primary composite endpoint of COVID-19 infection (admission to an intensive care unit [ICU], the use of mechanical ventilation, or death) (3, 6, 13–15). For example, Zhang et al. (7) reported that 70% of the COVID-19 patients were age>50 years older. Chen et al. (13) performed a retrospective, single-center study in Shanghai, China, and reported that age (OR = 1.06) is independently associated with admission to the ICU. Wu et al. (14) reported that older age (hazard ratio [HR] = 3.26, 95% CI = 2.08–5.11; HR = 6.17, 95% CI = 3.26–11.67, respectively), is a risk factor associated with the development of COVID-19 and eventually death. We found that the expression of ACE2 was highest in the >60 age group followed by the 40–60, 20–40, and <20 age groups. Moreover, Pearson's analysis, multivariate linear regression analysis, and logistic regression analysis also revealed that there is a statistically significant positive correlation between age and the expression of ACE2. This may explain the reason why there is an association between being older and susceptibility to infection with COVID-19 and the presence of a primary composite endpoint.

In addition, we found that smokers exhibit a significantly higher expression of ACE2 compared to non-smokers. Interestingly, Guan et al. (3) reported that both former smokers (49%) and current smokers (21.7%) are at a higher risk of developing severe disease compared to non-smokers (14.5%). Moreover, Cai et al. (11) also observed a significantly higher ACE2 gene expression in the lungs of former smokers compared to non-smokers, as well as a higher expression of ACE2 in current smokers compared to non-smokers. Our results suggested that smokers, especially males, may be more susceptible to infection with COVID-19. With regard to the association between cancer and susceptibility to infection with COVID-19, it has been reported in recent studies that breast, colorectal, and lung cancer may be associated with an increased expression of ACE2 (8, 10). Zheng et al. (16) reported that 8 (1%) of 1,590 COVID-19 cases had a history of cancer, which seems to be higher than the incidence of cancer in the overall Chinese population ([0.29%] per 100,000 people). Liang et al. (17) recently also suggested that patients with cancer might be at a higher risk of infection compared to those without. In this study, we also reported that subjects with cancer exhibit a higher expression of ACE2 compared to those without.

Furthermore, hypertension and cardiocerebrovascular diseases were found to cause a higher expression of ACE2 and, hence, a higher risk of infection. However, multivariate linear regression analysis showed no statistically significantly link between ACE2 and hypertension, ACE2 and cardiocerebrovascular diseases. Moreover, we did not observe any significant difference in the ACE2 gene expression between subjects with diabetes and those without, between drinkers and non-drinkers, and between subjects with different BMI values (<18.5, 18.5–24, 24–27, >27 kg/m^2). However, Rao et al. (8) showed that diabetes is associated with an increased expression of ACE2. One study (14) showed that, in subjects infected with COVID-19 who developed acute respiratory distress syndrome, compared to those who did not, more patients presented with hypertension (23/84 patients [27.4%] and 16/117 patients [13.7%]) and diabetes (16/84 patients [19.0%] patients and 6/117 patients [5.1%]). Our results are preliminary but are worthy of further studies.

Multiple previous studies have shown ACE2 expression in the lung, kidney, heart, testis, and small intestine of humans (10, 11, 18). As far as we known, blood cells are also the sources of the ACE2 mRNA. For example, Rutkowska-Zapała et al. (19) and Obitsu et al. (20) both reported that ACE2 mRNA was observed in human monocytes and their subsets. Moreover, apoptotic bodies, exosomes, or cast-off cells of endothelial cells contain mRNA that might contribute to the results. Further studies were needed.

In conclusion, we herein identified several demographic and clinical characteristics that may be causally related to the level of ACE2, the level of ACE2 is higher in females, older subjects, smokers, and subjects with cancer than in other subjects. Thus, gender, age, smoking habits, and cancer may provide valuable information for identifying susceptible populations.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Eye, Ear, Nose and Throat (Eye and ENT) Hospital of Fudan University, Shanghai, China, and was approved by the Ethics Committee of the same hospital. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

SL and WC conceived the study and participated in drafting the final manuscript. SL, JH, AZ, YH, MC, ZL, MS, and WC analyzed the data and completed the final draft of the manuscript. SL and JH prepared all the figures. All authors have read and approved the manuscript.

FUNDING

This work was supported by Shanghai Sailing Program (18YF1403500), Shanghai Municipal Commission of Health and Family Planning (20174Y0169), Shanghai Municipal Commission of Health and Family Planning (201840050), and Shanghai Science and Technology Committee Foundation Grant (19411964600).

REFERENCES

1. Lu R, Zhao X, Li J, Niu P, Yang B, Wu H, et al. Genomic characterisation and epidemiology of 2019 novel coronavirus: implications for virus origins and receptor binding. *Lancet*. (2020) 395:565–74. doi: 10.1016/S0140-6736(20)30251-8
2. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet*. (2020) 395:497–506. doi: 10.1016/S0140-6736(20)30183-5
3. Guan WJ, Ni ZY, Hu Y, Liang W-H, Qu C-Q, He J-X, et al. Clinical characteristics of coronavirus disease 2019 in China. *N Engl J Med*. (2020) 382:1708–20. doi: 10.1056/NEJMoa2002032
4. WHO. Available online at: https://www.who.int/docs/default-source/coronaviruse/situation-reports/20200325-sitrep-65-covid-19.pdf?sfvrsn=ce13061b_2 (accessed June 10, 2020).
5. Liu W, Tao ZW, Lei W, Yuan ML, Liu K, Zhou L, et al. Analysis of factors associated with disease outcomes in hospitalized patients with 2019 novel coronavirus disease. *Chin Med J*. (2020) 133:1032–8. doi: 10.1097/CM9.0000000000000775
6. Tian S, Hu N, Lou J, Chen K, Kang X, Xiang Z, et al. Characteristics of COVID-19 infection in Beijing. *J Infect*. (2020) 80:401–6. doi: 10.1016/j.jinf.2020.02.018
7. Zhang JJ, Dong X, Cao YY, Yuan YD, Yang YB, Yan YQ, et al. Clinical characteristics of 140 patients infected with SARS-CoV-2 in Wuhan, China. *Allergy*. (2020) 75:1730–41. doi: 10.1111/all.14238
8. Rao S, Lau A, So HC. Exploring diseases/traits and blood proteins causally related to expression of ACE2, the putative receptor of 2019-nCov: a mendelian randomization analysis. *medRxiv Preprint*. (2020). doi: 10.1101/2020.03.04.20031237
9. Wan Y, Shang J, Graham R, Baric RS, Li F. Receptor recognition by the novel coronavirus from Wuhan: an analysis based on decade-long structural studies of SARS coronavirus. *J Virol*. (2020) 94:e00127-20. doi: 10.1128/JVI.00127-20
10. Chen H, Xuan B, Yan Y, Zhu X, Shen C, Zhao G, et al. Profiling ACE2 expression in colon tissue of healthy adults and colorectal cancer patients by single-cell transcriptome analysis. *medRxiv Preprint*. (2020). doi: 10.1101/2020.02.15.20023457
11. Cai G. Bulk and single-cell transcriptomics identify tobacco-use disparity in lung gene expression of ACE2, the receptor of 2019-nCov. *medRxiv Preprint*. (2020). doi: 10.1101/2020.02.05.20020107
12. Li S, Li D, Shao M, Cao W, Sun X. Decreased serum levels of complement C3 reflect complement system dysregulation in patients with primary open-angle glaucoma: results from a pilot study. *J Glaucoma*. (2018) 27:761–8. doi: 10.1097/IJG.0000000000001014
13. Chen J, Qi T, Liu L, Ling Y, Qian Z, Li T, et al. Clinical progression of patients with COVID-19 in Shanghai, China. *J Infect*. (2020). 80:e1–6. doi: 10.1016/j.jinf.2020.03.004
14. Wu C, Chen X, Cai Y, Xia J, Zhou X, Xu S, et al. Risk factors associated with acute respiratory distress syndrome and death in patients with coronavirus disease 2019. Pneumonia in Wuhan, China. *JAMA Intern Med*. (2020). 180:1–11. doi: 10.1001/jamainternmed.2020.0994
15. Wang Z, Yang B, Li Q, Wen L, Zhang R. Clinical features of 69 cases with coronavirus disease 2019 in Wuhan, China. *Clin Infect Dis*. (2020). 16:ciaa272. doi: 10.1093/cid/ciaa272
16. Zheng RS, Sun KX, Zhang SW. Report of cancer epidemiology in China, 2015. *Zhonghua Zhong Liu Za Zhi*. (2019) 41:19–28. doi: 10.3760/cma.j.issn.0253-3766.2019.01.005
17. Liang W, Guan W, Chen R, Wang W, Li J, Xu K, et al. Cancer patients in SARS-CoV-2 infection: a nationwide analysis in China. *Lancet Oncol*. (2020) 21:335–7. doi: 10.1016/S1470-2045(20)30096-6
18. Hamming I, Timens W, Bulthuis ML, Lely AT, Navis G, van Goor H. Tissue distribution of ACE2 protein, the functional receptor for SARS coronavirus. A first step in understanding SARS pathogenesis. *J Pathol*. (2004) 203:631–7. doi: 10.1002/path.1570
19. Rutkowska-Zapała M, Suski M, Szatanek R, Lenart M, Weglarczyk K, Olszanecki R, et al. Human monocyte subsets exhibit divergent angiotensin I-converting activity. *Clin Exp Immunol*. (2015) 181:126–32. doi: 10.1111/cei.12612
20. Obitsu S, Ahmed N, Nishitsuji H, Hasegawa A, Nakahama K, Morita I, et al. Potential enhancement of osteoclastogenesis by severe acute respiratory syndrome coronavirus 3a/X1 protein. *Nephrol Dialy Transpl*. (2018) 34–43. doi: 10.1007/s00705-009-0472-z.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cell and Developmental Biology

ORIGINAL RESEARCH
published: 19 August 2020
doi: 10.3389/fcell.2020.00783

Single-Cell RNA-seq Identifies Cell Subsets in Human Placenta That Highly Expresses Factors Driving Pathogenesis of SARS-CoV-2

Nancy Ashary[1†], Anshul Bhide[1†], Priyanka Chakraborty[2], Stacy Colaco[1], Anuradha Mishra[1], Karisma Chhabria[1], Mohit Kumar Jolly[2] and Deepak Modi[1]*

[1] Molecular and Cellular Biology Laboratory, ICMR-National Institute for Research in Reproductive Health, Indian Council of Medical Research (ICMR), Mumbai, India, [2] Center for BioSystems Science and Engineering, Indian Institute of Science, Bengaluru, India

OPEN ACCESS

Edited by:
Tatsuo Shioda,
Osaka University, Japan

Reviewed by:
Ramanuj Dasgupta,
Genome Institute of Singapore, Singapore
Surendra Sharma,
Women & Infants Hospital of Rhode Island, United States

***Correspondence:**
Deepak Modi
deepaknmodi@yahoo.com;
modid@nirrh.res.in

† These authors have contributed equally to this work

Specialty section:
This article was submitted to Molecular Medicine, a section of the journal Frontiers in Cell and Developmental Biology

Received: 09 May 2020
Accepted: 27 July 2020
Published: 19 August 2020

Citation:
Ashary N, Bhide A, Chakraborty P, Colaco S, Mishra A, Chhabria K, Jolly MK and Modi D (2020) Single-Cell RNA-seq Identifies Cell Subsets in Human Placenta That Highly Expresses Factors Driving Pathogenesis of SARS-CoV-2. Front. Cell Dev. Biol. 8:783. doi: 10.3389/fcell.2020.00783

Infection by the Severe Acute Respiratory Syndrome-Coronavirus-2 (SARS-CoV-2) results in the novel coronavirus disease COVID-19, which has posed a serious threat globally. Infection of SARS-CoV-2 during pregnancy is associated with complications such as preterm labor and premature rupture of membranes, and a proportion of neonates born to infected mothers are also positive for the virus. During pregnancy, the placental barrier protects the fetus from pathogens and ensures healthy development. To predict if the placenta is permissive to SARS-CoV-2, we utilized publicly available single-cell RNA-seq data to identify if the placental cells express the necessary factors required for infection. SARS-CoV-2 binding receptor *ACE2* and the S protein priming protease *TMPRSS2* are co-expressed by a subset of syncytiotrophoblasts (STB) in the first trimester and extravillous trophoblasts (EVT) in the second trimester human placenta. In addition, the non-canonical receptor *BSG/CD147* and other proteases (*CTSL, CTSB*, and *FURIN*) are detected in most of the placental cells. Other coronavirus family receptors (*ANPEP* and *DPP4*) were also expressed in the first and second trimester placental cells. Additionally, the term placenta of multiple species including humans expressed *ACE2*, *DPP4*, and *ANPEP* along with the viral S protein proteases. The *ACE2*- and *TMPRSS2*-positive (*ACE2* + *TMPRSS2* +) placental subsets expressed mRNA for proteins involved in viral budding and replication. These cells also had the mRNA for proteins that physically interact with SARS-CoV-2 in host cells. Further, we discovered unique signatures of genes in *ACE2* + *TMPRSS2* + STBs and EVTs. The *ACE2* + *TMPRSS2* + STBs are highly differentiated cells and express genes involving mitochondrial metabolism and glucose transport. The second trimester *ACE2* + *TMPRSS2* + EVTs are enriched for markers of endovascular trophoblasts. Both these subtypes abundantly expressed genes in the Toll-like receptor pathway. The second trimester EVTs are also enriched for components of the JAK-STAT pathway that drives inflammation. We carried out a systematic review and identified that in 12% of pregnant women with COVID-19, the placenta was infected with SARS-CoV-2, and

the virus was detected in STBs. To conclude, herein we have uncovered the cellular targets for SARS-CoV-2 entry and have shown that these cells can potentially drive viremia in the developing human placenta. Our results provide a basic framework toward understanding the paraphernalia involved in SARS-CoV-2 infections in pregnancy.

Keywords: placenta, trophoblast, SARS-CoV-2, coronaviruses, receptors, single-cell RNA-seq, inflammation, COVID-19

INTRODUCTION

Epidemiologic evidence indicates that pregnant women are at higher risk of severe illness and mortality from viral infections such as influenza, Ebola and Lassa fever (Silasi et al., 2015). Certain viral infections during pregnancy can lead to several adverse pregnancy outcomes such as spontaneous abortion, mother-to-child transmission resulting in congenital viral syndromes, still-births and intrauterine fetal deaths (Silasi et al., 2015; Arora et al., 2018). Furthermore, viral infection also predisposes the pregnancy toward preterm birth, which has major long-term health implications for the newborn. Thus, understanding the health risks of viral infections during pregnancy is vital for designing appropriate approaches for its clinical management. The importance of understanding the role of viral infection during pregnancy gains further relevance as we are confronted with newer pandemics, which may affect the pregnant mother and the fetus.

Coronaviruses (CoV) are positive-sense RNA viruses that, upon zoonotic transmission, lead to respiratory disease in humans and some animals. Previous outbreaks of zoonotic coronaviruses, including the severe acute respiratory syndrome (SARS)-CoV and the Middle East respiratory syndrome (MERS)-CoV, have proven to be of great public health concern. Another outbreak of severe acute respiratory syndrome called coronavirus disease-2019 (COVID-19) has been recently reported, which is due to infection by a novel coronavirus termed SARS-CoV-2 (Zhu et al., 2020). The infection has spread rapidly worldwide due to high human-to-human transmission, resulting in a public health emergency of international concern (Bharti et al., 2020; Gupta et al., 2020; Li R. et al., 2020). Presently, there are no specific treatments available for COVID-19, and there is an urgent need to identify the drugs and vaccines targeted against this virus (Prajapat et al., 2020).

Since SARS-CoV-2 has not been detected in humans before, limited information is available about its health effects; negligible information is available for pregnant women. In pregnant women, COVID-19 is associated with severe pregnancy complications such as preterm labor and premature rupture of membranes (Gajbhiye et al., 2020). Furthermore, a proportion of neonates born to mothers with COVID-19 are positive for the virus, suggesting the possibility of vertical transmission through the placental barrier (Gajbhiye et al., 2020; Knight et al., 2020).

The placenta is a highly specialized organ that maintains the equilibrium between immunological and biochemical factors required for fetal development (Deshpande and Balasinor, 2018). It also acts as a barrier for vertical transmission of pathogens (Maltepe and Fisher, 2015; Burton et al., 2016). However, some viruses such as Zika can infect the placental cells via receptors on trophoblasts, leading to fetal malformation and pregnancy complications (Hirsch et al., 2018; Tabata et al., 2018). Interestingly, placental and amniotic fluid infections of SARS-CoV-2 have been reported (Baud et al., 2020; Zamaniyan et al., 2020). For SARS-CoV-2 to be able to infect the placenta, the host cells must harbor the necessary receptors and virus-processing machinery. It has been shown that SARS-CoV-2 binds and infects host cells by utilizing the membrane-bound Angiotensin-Converting Enzyme II (ACE2), which is considered its canonical mode of action (Jagtap et al., 2020; Letko et al., 2020; Shang et al., 2020). In addition, SARS-CoV-2 binds to CD147/Basigin (BSG) on the cell surface that may act as an alternate non-canonical receptor (Wang et al., 2020). Upon receptor binding, the viral-encoded S protein requires cleavage by host proteases for efficient membrane fusion. The main host protease that mediates S protein priming and initiates viral entry is the Type II transmembrane serine protease TMPRSS2 (Hoffmann et al., 2020). The endosomal protease cathepsin L (CTSL) can also enhance viral entry (Hoffmann et al., 2020). Thus, the presence of such receptors and S protein primer proteases in host cells is a key determinant of SARS-CoV-2 infection. Indeed, expression of *ACE2* and *TMPRSS2* have been detected in lung airway cells and the upper respiratory epithelium, the primary site of SARS-CoV-2 action (Sungnak et al., 2020; Ziegler et al., 2020). Beyond respiratory distress, some patients with SARS-CoV-2 viremia develop multiple organ injuries, and cells of these tissues also express *ACE2* and *TMPRSS2* (Qi et al., 2020; Seow et al., 2020; Zou et al., 2020).

The binding of enveloped viruses like SARS-CoV-2 to its receptors results in events related to membrane fusion and/or endocytosis followed by establishment of the primary infection. Following its entry and uncoating, coronavirus replication is initiated by translation of its non-structural proteins including the replicases that allow viral RNA synthesis and capping. This course requires a network of host factors to create an optimal environment for facilitating viral entry, gene expression, RNA synthesis and virus release (de Wilde et al., 2018). Further, most enveloped viruses bud at the plasma membrane by recruiting the host endosomal sorting complex required for transport (ESCRT) machinery (Ahmed et al., 2019; Gatta and Carlton, 2019). While the precise host proteins in SARS-CoV-2 entry and replication are not yet understood, its host interactome has been characterized (Gordon et al., 2020). The host proteins that interact with SARS-CoV-2 are involved in endocytosis and replication of viruses (Gordon et al., 2020). Thus, elucidating tissue and cell-type-specific host machinery that not only mediate viral entry but also replication and budding from the host cell is essential to understand the pathogenesis of SARS-CoV-2 infection.

Single-cell RNA sequencing (scRNA-seq) of different tissues has transformed our ability to map the types, subsets and states of cells in healthy and diseased conditions in an unprecedented manner (Sharma et al., 2018; Szabo et al., 2019; Iyer et al., 2020). Recently, scRNA-seq has been applied to expand our understanding of the cellular landscape during viral infection including that of SARS-CoV-2 (Russell et al., 2018; Galinato et al., 2019; Liao et al., 2020). scRNA-seq has also been used in the identification of various tissues and cells that are potential targets of SARS-CoV-2, and these studies have immensely contributed toward expanding our understanding of the molecular characteristics of the host cells that are targets of viral infection (Colaco et al., 2020; Lukassen et al., 2020; Qi et al., 2020; Seow et al., 2020; Singh et al., 2020; Sungnak et al., 2020; Zhang et al., 2020).

To gain an insight into the pathogenesis of SARS-CoV-2 infection during pregnancy, it is essential to identify and characterize the placental cell types that express the viral receptors ACE2 and BSG/CD147, along with the proteases TMPRSS2 and CTSL. Recent studies have reported *ACE2*-positive cells in early embryonic trophoblasts as well as first trimester human placenta (Colaco et al., 2020; Li M. et al., 2020; Singh et al., 2020). ACE2 protein is also detected in term human placenta[1]. Studies have also shown that *BSG*/*CD147* is expressed in first trimester human trophoblasts and gestational day 18 mouse placenta (Bharadwaj et al., 2011; Lee et al., 2013). However, information regarding the cells co-expressing various coronavirus receptors and S protein proteases as well as their detailed characteristics in the placenta is unknown. Herein (Ashray et al., 2020), we surveyed the publicly available scRNA-seq data of human placenta for the expression of the SARS-CoV-2 receptors *ACE2* and *BSG*/*CD147*, along with the S protein proteases *TMPRSS2* and *CTSL*. We also surveyed the placental cells for the expression of *DPP4* and *ANPEP*, which are utilized by MERS-CoV and CoV-229E, respectively. The study also aimed to characterize the *ACE2*- and *TMPRSS2*-positive placental cells for their possible roles in viral endocytosis, replication, SARS-CoV-2 interactions and viral budding. The results reveal that placental cells are potential targets for SARS-CoV-2 infection.

MATERIALS AND METHODS

To identify the population of human placental cells that express *ACE2, BSG, TMPRSS2*, and *CTSL* at single-cell resolution, we analyzed scRNA-seq data of first and second trimester human placenta (Liu et al., 2018) [Accession number GSE89497]. This dataset is derived out of 7 first trimester (8 weeks) placentae and 1 second trimester placenta (24 weeks) in which single cells were isolated by enzymatic digestion followed by enrichment of cells using a combination of methods. Extravillous trophoblasts (EVTs) were enriched by Magnetic Activated Cell Sorting (MACS) using anti-HLA-G antibody, cytotrophoblasts (CTBs) were enriched using anti-CDH1 antibody, and syncytiotrophoblasts (STBs) were manually sorted. The HLA-G- and CDH1-negative fraction was designated to be villous stromal cells (STR). To isolate EVTs from the placenta at 24 weeks, the basal plate was dissected from the villi of the placenta, and the single cells were prepared using enzymatic digestion followed by MACS using anti-HLA-G antibody. We deliberately chose this "index sorted" dataset over the unbiased agnostic scRNA-seq datasets since our major focus was to identify the cellular targets of SARS-CoV-2 specifically in the placenta, and such *a priori* approach allows analysis of homogeneous cell populations and accurate linking of rare transcripts with the index sorted cells.

To understand changes in the expression of *ACE2, BSG, TMPRSS2*, and *CTSL* in the second and third trimester placenta, bulk RNA-seq data was analyzed [Accession number GSE124282]. This dataset is derived out of 4 second trimester and term human placentae (Wang et al., 2019). Bulk RNA-seq data (Armstrong et al., 2017) was also analyzed of term placenta from human, cow, dog, armadillo, elephant, opossum, mouse and bonobo samples [Accession number GSE79121] to understand if the expression of the SARS-CoV-2 receptors in the term placenta is evolutionarily conserved. Pseudo-bulk scRNA-seq data of human first trimester decidua (Suryawanshi et al., 2018) was then analyzed to understand the distribution of *ACE2, BSG, TMPRSS2*, and *CTSL* in different maternal cell populations.

We profiled the mRNA levels of 27 host proteins involved in human ESCRT for viruses (Ahmed et al., 2019) and mRNA levels of 30 proteins involved in viral replication (de Wilde et al., 2018) in placental cells that co-express *ACE2* and *TMPRSS2* in first and second trimester (**Supplementary Table 1**). We also analyzed the transcript profiles of 332 human proteins that physically interact with SARS-CoV-2 in placental cells that co-express *ACE2* and *TMPRSS2* (Gordon et al., 2020). To guard against viral infection, host cells express a plethora of genes that sense the presence of the virus on the cell surface, in cytosol and in endosomes. This in turn activates the host defense mechanisms to limit or eliminate viral infection and restore tissue homeostasis. We profiled the expression of 487 host viral response genes in placental cells that co-express *ACE2* and *TMPRSS2* (**Supplementary Table 1**).

To characterize the trophoblast cells that co-express *ACE2* and *TMPRSS2* as well as their counterparts that do not express both of these genes; we carried out pseudo-bulk analysis of *ACE2*- and *TMPRSS2*-positive (*ACE2* + *TMPRSS2* +) and *ACE2*- and *TMPRSS2*-negative *(ACE2–TMPRSS2–)* trophoblast cells. Single-cell data for *ACE2* + *TMPRSS2* + and *ACE2–TMPRSS2–* cells was independently aggregated and the mean TPM values were computed. The data was filtered for genes whose mean values were $\geq$0.1 TPM and the ratio of the mean value in *ACE2* + *TMPRSS2* + cells over *ACE2–TMPRSS2–* cells was calculated. The genes that had a ratio of $\geq$1.5 or $\leq$0.5 and *p*-value of $<$0.05 were filtered and the data was deconvoluted for single cells. Gene ontology (GO) analysis was performed using the PANTHER database and over-representation tests were performed using reference genes of PANTHER pathways[2].

To determine if SARS-CoV-2 is detected in the placenta, one author (AB) carried out a systematic review with the keywords

[1]https://www.proteinatlas.org/ENSG00000130234-ACE2/tissue/placenta

[2]http://www.pantherdb.org/

"placenta and SARS-CoV-2," "COVID-19 and placenta," "SARS-CoV-2 and pregnancy," "coronaviruses" and "pregnancy." The primary outcome was to determine the number of cases reporting the presence of SARS-CoV-2 in placental tissue; the secondary outcome was to determine the placental cell types positive for SARS-CoV-2. Searches were carried out in PubMed, Google Scholar, MedRxiv, bioRxiv and other preprint databases. Articles reporting primary data in which detection of SARS-CoV-2 was carried out by either RT-PCR and/or by immunohistochemistry or electron microscopy were included. Reviews, blog and newspaper reports were excluded. Data was entered in tabular format and was independently verified by another author (DM).

All the data was processed using R Studio version 3.6.2. Heatmaps were plotted using the heatmap.2 function from the gplots R package and the pheatmap R package. Uniform Manifold Approximation and Projection (UMAP) analysis was performed using the UMAP 0.2.6.0 R package. To identify the cell clusters, 500 genes with high SD and average log2-transformed expression >1 were selected. Next, these 500 genes were given as an input to UMAP for calculating the projections of all cells. Statistical analysis was done using the Welch's *t*-test and the graphs were plotted in GraphPad Prism version 8.0.

RESULTS

Trophoblast Cells Express mRNA for SARS-CoV-2 Receptors and Spike Protein Processing Enzymes

The human placenta is characterized by four distinct cell lineages: extravillous trophoblasts (EVT), cytotrophoblasts (CTB), syncytiotrophoblasts (STB), and villous stromal cells (STR). To understand the distribution of the SARS-CoV-2 receptors in these cell types, we analyzed publicly available single-cell transcriptome data from human placenta. The results revealed that *ACE2*, *BSG*, *TMPRSS2* and *CTSL* were expressed in all the cell types of first trimester placenta; however, not every cell of each type expressed these genes (**Figure 1A**). As evident, STBs represented the highest proportion of *ACE2*-expressing cells (39%) in first trimester placenta, whereas only 2% of EVTs had *ACE2* expression. *BSG* was abundantly expressed in almost all the cells (96–100%) of EVT, CTB, STB, and STR of the first trimester (**Supplementary Table 2**). Very few cells of the human placenta expressed *TMPRSS2*. The highest *TMPRSS2* expression was detected in STBs of first trimester placenta (23%), whereas only 1% of CTBs had *TMPRSS2* expression (**Supplementary Table 2**). *CTSL* was expressed in nearly all STBs, CTBs, EVTs and STRs of first trimester placenta (**Figure 1A**). The numbers of cells that express these genes individually are given in **Supplementary Table 2**.

We then compared the expression of these genes in EVTs of the first trimester and second trimester human placenta (**Figure 1B**). 2% of first trimester EVTs and 62% of second trimester EVTs expressed *ACE2*. Similarly, the numbers of EVTs expressing *TMPRSS2* also increased in the second trimester as compared to the first trimester (2 vs. 19%) (**Supplementary Table 2**). In both cases, the increase was statistically significant (p-value ≤ 0.001). Nearly all EVT cells in the first and the second trimesters expressed *BSG* and *CTSL* (**Supplementary Table 2**). *BSG* expression was significantly reduced in second trimester EVTs as compared to the first trimester (p-value ≤ 0.001); the expression of *CTSL* was significantly higher in second trimester EVTs as compared to first trimester EVTs (p-value ≤ 0.001) (**Figure 1B**).

We also studied the expression of other SARS-CoV receptors *DPP4* and *ANPEP*, which are utilized by MERS-CoV and CoV-229E, respectively. *ANPEP* was detected in the EVTs, CTBs and STRs but not in the STBs of the first trimester placenta. *DPP4*, on the other hand, was expressed by all CTBs, STBs, EVTs, and STRs (**Supplementary Figure 1A**). As compared to first trimester EVTs, the levels of *ANPEP* significantly decreased (p-value ≤ 0.001), while those of *DPP4* significantly increased (p-value ≤ 0.001) in the second trimester EVTs (**Supplementary Figure 1B**).

Bulk transcriptome analysis revealed that, in comparison to the second trimester, the levels of *ACE2, BSG*, and *DPP4* were reduced in the term placenta; however, a statistically significant reduction was observed only in the case of *BSG* (p-value ≤ 0.05). Although the levels of *TMPRSS2* and *ANPEP* were higher in the term placenta as compared to second trimester placenta, this increase was not statistically significant. *CTSL* levels were similar in the second trimester and term placentae (**Supplementary Figure 2A**). In the absence of publicly available index sorted scRNA-seq data of term placenta, we cannot comment on the cell types that express these genes.

The expression of coronavirus receptors and spike protein proteases was compared in term placenta of different species (**Supplementary Figure 2B**). *ACE2* transcripts were detected in the placenta of most mammals except bonobo. *BSG* mRNA was expressed in term placenta of all the species, albeit at varying levels. *TMPRSS2* mRNA was expressed in the placenta of most species except dog, armadillo, and elephant. *CTSL* mRNA was detected in human, cow, dog, mouse, and bonobo placentae. *ANPEP* was only expressed in human, armadillo and mouse placentae, and *DPP4* was expressed in placenta of all species except mouse.

To determine if endometrial cells express these genes, we analyzed pseudo-bulk data of the first trimester feto-maternal interface (**Supplementary Figure 3**). Expression of *ACE2* was detected in smooth muscle cells, and a low abundance of *ACE2* transcripts was also detected in decidual stromal cells and fibroblasts, vascular endothelial cells and NK cells. *TMPRSS2* transcripts were detected in endometrial epithelial cells and lymphatic endothelial cells. *BSG* and *CTSL* were detected in all the maternal cells of the first trimester feto-maternal interface.

Co-expression of mRNA of SARS-CoV-2 Receptors and Spike Protein Processing Enzymes in Human Placental Cells

Uniform Manifold Approximation and Projection revealed distinct clusters of CTBs and STBs, and EVTs in the

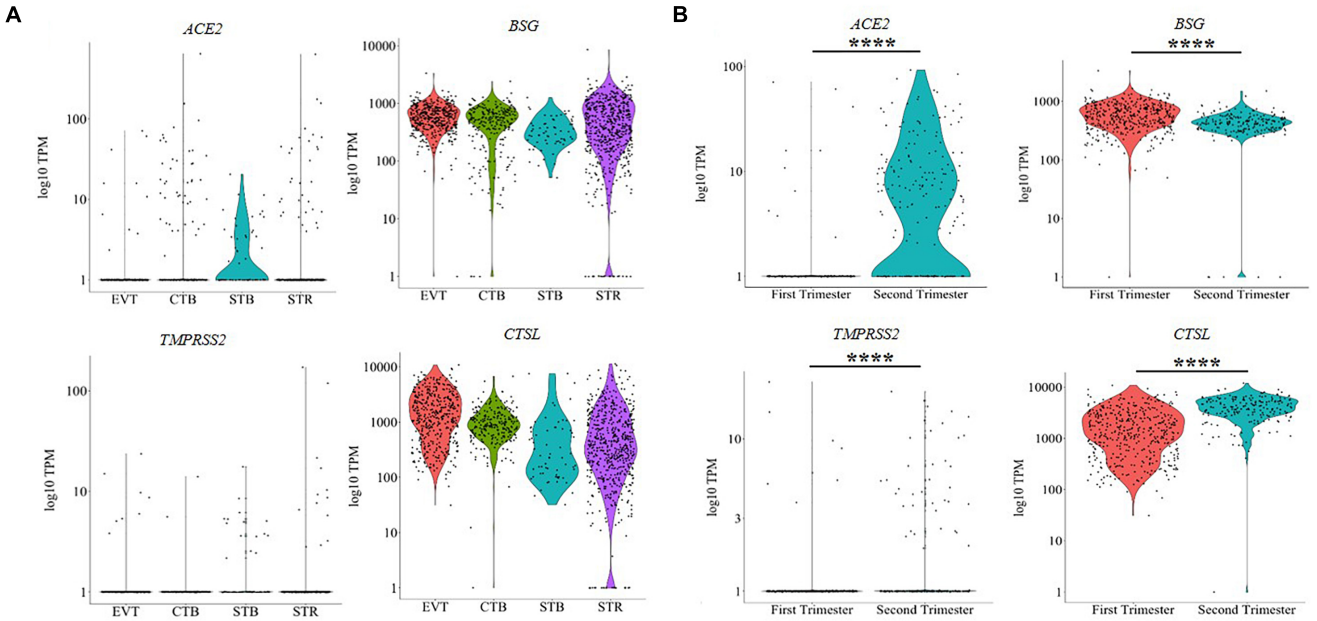


FIGURE 1 | Trophoblast cells express mRNA for SARS-CoV-2 receptors and spike protein processing enzymes. **(A)** mRNA level of SARS-CoV-2 receptors (*ACE2* and *BSG*) and spike protein primer enzymes (*TMPRSS2* and *CTSL*) in different cell types of first trimester human placenta [EVT (n = 440), CTB (n = 248), STB (n = 64), STR (n = 615)]. **(B)** Comparison of the mRNA levels of *ACE2, BSG, TMPRSS2*, and *CTSL* in EVT of first (n = 440 cells) and second (n = 200 cells) trimester human placenta. Each dot represents data of a single cell, the *Y* axis represents $\log_{10}$ Transcripts Per Million (TPM). Bar within the cluster denotes mean. Horizontal black bars denote significantly different values (**** indicates p-value $\leq$ 0.0001). Data was extracted from single-cell RNA-seq of human placenta (Liu et al., 2018) [Accession number GSE89497]. EVT, extravillous trophoblast; CTB, cytotrophoblast; STB, syncytiotrophoblast; STR, villous stromal cell.

first trimester placenta; the second trimester EVTs also formed an independent cluster (**Figure 2A**). Some STR cells clustered independently while others clustered with the different trophoblast cell types, suggesting that the STRs are not a pure population. This finding was expected since the STRs were the post-enrichment leftover fractions of CTBs, STBs and EVTs. The data on this population was hence excluded in further analysis. SARS-CoV-2 infection in host cells requires coordinated expression of the entry receptor *ACE2* and S protein primer *TMPRSS2*. UMAP analysis revealed that a subset of CTBs, STBs and EVTs co-expressed both *ACE2* and *TMPRSS2* (**Figure 2B**).

We next evaluated CTBs, STBs and EVTs co-expressing *ACE2, TMPRSS2*, *BSG*, and *CTSL* in different combinations (**Figure 3**). The results revealed that a subset of STBs (14%) in the first trimester placenta co-expressed *ACE2* and *TMPRSS2* (**Supplementary Table 3**). No other cell types in first trimester placenta expressed this receptor and S protein primer protease pair, although there were cells expressing *ACE2* (**Figure 3**). However, 15% of EVTs in the second trimester placenta co-expressed *ACE2* and *TMPRSS2* (**Supplementary Table 3**).

In the first trimester placenta, all the *ACE2*-positive trophoblast subtypes co-expressed *BSG* and *CTSL*, and all the *BSG*-positive cells co-expressed *CTSL*. All the *ACE2*-positive second trimester EVTs co-expressed *BSG* and *CTSL*, and all the *BSG*-positive cells co-expressed *CTSL* (**Figure 3**). The absolute numbers and percentages of the co-expressing cells are given in **Supplementary Table 3**.

ACE2 + *TMPRSS2* + First Trimester Syncytiotrophoblast Cells Are Highly Differentiated and Express the Machinery for Viral Endocytosis, Replication and Budding

Since only STBs co-expressed *ACE2* and *TMPRSS2* in the first trimester, we carried out an in-depth characterization of these cells. 14% of the total STB population of the first trimester placenta expressed both *ACE2* and *TMPRSS2*, 52% did not express either, and the rest of the cells expressed either *ACE2* or *TMPRSS2* (**Supplementary Table 3**). We compared the expression profiles of classical STB genes between the *ACE2* and *TMPRSS2* co-expressing cells and *ACE2*- and *TMPRSS2*-negative STBs. We observed that both cell types abundantly expressed the transcripts for human chorionic gonadotropin beta 5 (*CGB5*) and somatomammotropin [placental lactogen (*CSH1*)], as well as steroid hormone biosynthesis enzymes (*HSD17B1* and *CYP19A1*). Further, both these subsets of STBs abundantly expressed the other putative SARS-CoV-2 S protein primers *FURIN* and Cathepsin B (*CTSB*) (**Supplementary Figure 4**).

We next characterized the transcriptome differences between the *ACE2* + *TMPRSS2* + versus the *ACE2*–*TMPRSS2*– STB cells. Pseudo-bulk analysis identified 817 genes (including *ACE2* and *TMPRSS2*) between these two cell types (**Supplementary Table 4**). Of these, 444 were over represented while the others were under represented in the *ACE2* + *TMPRSS2* + cells as compared to *ACE2*–*TMPRSS2*– cells. These genes were

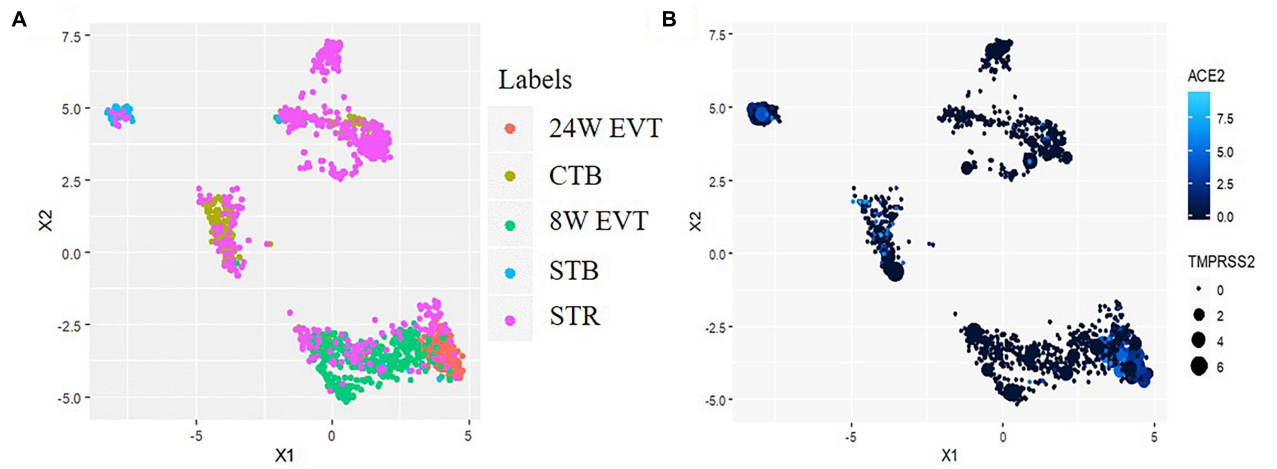


FIGURE 2 | Uniform Manifold Approximation and Projection (UMAP) plot illustrating cell clusters and *ACE2* & *TMPRSS2* expressing cells in the human placenta. Data was extracted from single-cell RNA-seq of human placenta (Liu et al., 2018) [Accession number GSE89497]. **(A)** Clusters consist of three trophoblast lineages (EVT, extravillous trophoblast; CTB, cytotrophoblast; STB, syncytiotrophoblast) and the villous stromal cells (STR). **(B)** The points colored blue are the cells co-expressing *ACE2* and *TMPRSS2*. In each blue point, the intensity of the color represents *ACE2* expression and the size of the point represents the extent of *TMPRSS2* expression.

heterogeneously expressed in the *ACE2*–*TMPRSS2*– STBs, while most *ACE2* + *TMPRSS2* + cells uniformly expressed these genes (**Figure 4A**). The biological processes enriched by these genes included regulation of G1/S cell-cycle checkpoints, actin polymerization/depolymerization, regulation of mitochondrial membrane permeability and electron-transport-coupled ATP synthesis, monosaccharide transport and unfolded protein response (**Figure 4B**). Most of the *ACE2* + *TMPRSS2* + cells significantly overexpressed the transcription factor *OVOL1* (a terminal STB differentiation marker), and the glucose transport regulators *GPC3* and *SLC2A9* (p-value $\leq$ 0.05). The expression of *ACTN1* (an actin binding protein) was significantly downregulated in *ACE2* + *TMPRSS2* + versus *ACE2*–*TMPRSS2*– STBs (p-value $\leq$ 0.05) (**Figure 4C**).

We next analyzed the mRNA levels of 27 genes involved in human ESCRT of viruses and 30 host genes involved in SARS-CoV replication in *ACE2*- and *TMPRSS2*-positive STB cells. All the *ACE2* + *TMPRSS2* + STBs uniformly expressed most of these genes (**Figure 4A**); however, the other cells showed heterogeneous expression across the different subtypes (**Supplementary Figure 5**). We also analyzed the mRNA levels of 332 host proteins that are known to interact with SARS-CoV-2 and found that there was minimal heterogeneity in expression of these genes in the first trimester STBs (**Figure 4D**) as compared to other cell types (**Supplementary Figure 5**).

Second Trimester *ACE2* + *TMPRSS2* + Cells Are Invasive Extravillous Trophoblasts and Express Markers of Endovascular Trophoblasts

Amongst the second trimester EVTs, 15% of cells were *ACE2* + *TMPRSS2* + while 33% did not express either of the transcripts (**Supplementary Table 3**). Two populations of second trimester EVTs are reported and characterized by the expression of TAC3. Type 1 EVTs are TAC3-high and express genes involved in migration and invasion; type 2 EVTs are TAC3-low cells that express genes involved in cell proliferation (Liu et al., 2018). In addition to TAC3, the type 1 EVTs also express JAM2, SERPENIN1 and PRG2 (Liu et al., 2018). We observed that the levels of *TAC3* were marginally but not significantly higher in *ACE2* + *TMPRSS2* + EVTs (**Supplementary Figure 6A**), and the mRNA levels of other genes were identical in *ACE2* + *TMPRSS2* + EVTs as compared to cells not expressing either of the two genes (*ACE2*– *TMPRSS2*–) (**Supplementary Figure 6A**). Principal component analysis did not reveal major differences in the transcriptome of the *ACE2* + *TMPRSS2* + and *ACE2*–*TMPRSS2*– cells (**Supplementary Figure 6B**). We compared the expression profiles of classical EVT genes between the *ACE2* + *TMPRSS2* + and *ACE2*–*TMPRSS2*– cells and observed that both the cell types abundantly expressed the transcripts for *HLA-G* and *ITGB1* (**Supplementary Figure 6C**). Both these subsets of EVTs also abundantly expressed other SARS-CoV-2 S protein primer proteins *FURIN* and *CTSB* of which the levels of *FURIN* were significantly higher (p-value $\leq$ 0.05) in *ACE2* + *TMPRSS2* + EVTs as compared to *ACE2*–*TMPRSS2*– EVTs (**Supplementary Figure 6C**).

To characterize if there are any specific classes of genes differentially abundant between the *ACE2* + *TMPRSS2* + versus the *ACE2*–*TMPRSS2*– EVT cells, pseudo-bulk analysis was carried out. There were 983 differentially abundant genes (including *ACE2* and *TMPRSS2*) between these two cell types (**Supplementary Table 5**) of which 931 were overrepresented and 52 were underrepresented in the *ACE2* + *TMPRSS2* + cells as compared to *ACE2*–*TMPRSS2*– cells. Further, these genes were heterogeneously expressed in the *ACE2*–*TMPRSS2*– EVTs while most *ACE2* + *TMPRSS2* + cells uniformly expressed

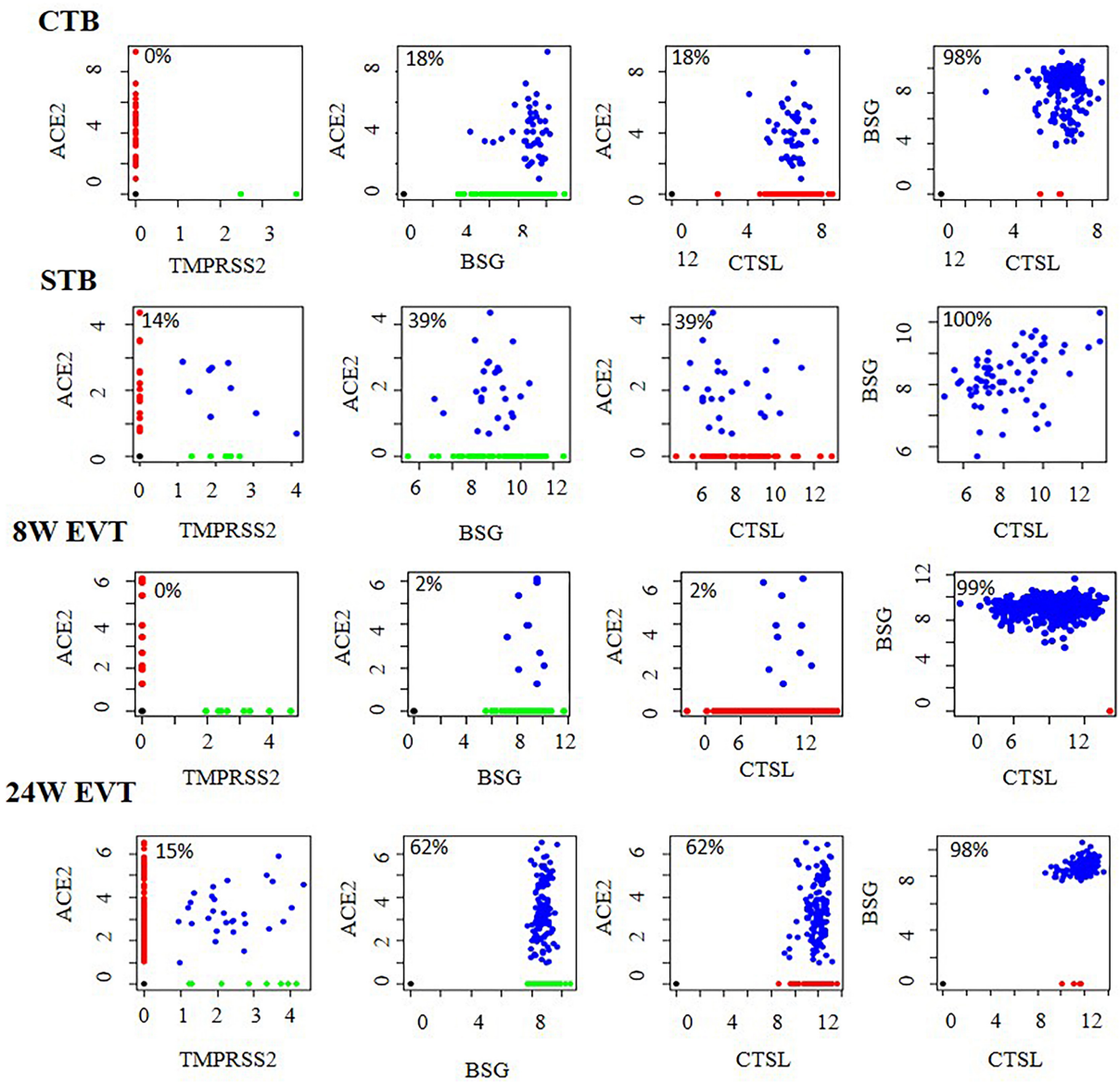


FIGURE 3 | Co-expression of mRNA of SARS-CoV-2 receptors and spike protein processing enzymes in human placental cells. Co-expression of *ACE2* and *TMPRSS2*, *ACE2* and *BSG, ACE2* and *CTSL*, and *BSG* and *CTSL* in STB (n = 64), CTB (n = 248), first trimester EVT (8W EVT, n = 440) and second trimester EVT (24W EVT, n = 200). Each dot represents data of a single cell. Values in each box are the percentage of co-expressing cells. Co-expressing cells are blue; single-positive cells are red and green. X and Y axes represent $\log_2$ Transcripts Per Million (TPM) values for that gene. Data was extracted from single cell RNA-seq of human placenta (Liu et al., 2018) [Accession number GSE89497]. EVT, extravillous trophoblast; CTB, cytotrophoblast; STB, syncytiotrophoblast.

these genes (**Figure 5A**). Most of these differentially abundant genes enriched several GO biological processes such as viral entry, release and intracellular transport. The other enriched GO biological processes were nucleic acid replication, epithelial morphogenesis and cell migration (**Figure 5B**).

The *ACE2* + *TMPRSS2* + cells significantly overexpressed the markers of endovascular trophoblasts *CDH5, VCAM*, *CCR1* and *CD59* (p-value $\leq$ 0.05) (**Figure 5C**). These cells also significantly overexpressed *OVOL2,* the marker of terminally differentiated EVTs, and the invasion-related marker *AKT1* (p-value $\leq$ 0.05) (**Figure 5C**). *ICAM* and *GJA5* are markers for EVTs in anchoring cell columns, and their levels were identical in the *ACE2* + *TMPRSS2* + and the *ACE2*–*TMPRSS2*– EVT cells.

Analysis of the mRNA levels of 27 genes involved in human ESCRT of viruses and 30 host genes involved in SARS-CoV replication in *ACE2*- and *TMPRSS2*-positive cells at single-cell resolution revealed that all the *ACE2* + *TMPRSS2* + EVTs uniformly expressed most of these genes (**Figure 5D**), while the first trimester EVTs that had no *ACE2* + *TMPRSS2* + cells had a very heterogeneous expression of these genes (**Supplementary Figure 5**). We also analyzed the mRNA levels of 332 host proteins that interact with SARS-CoV-2 and observed that almost all

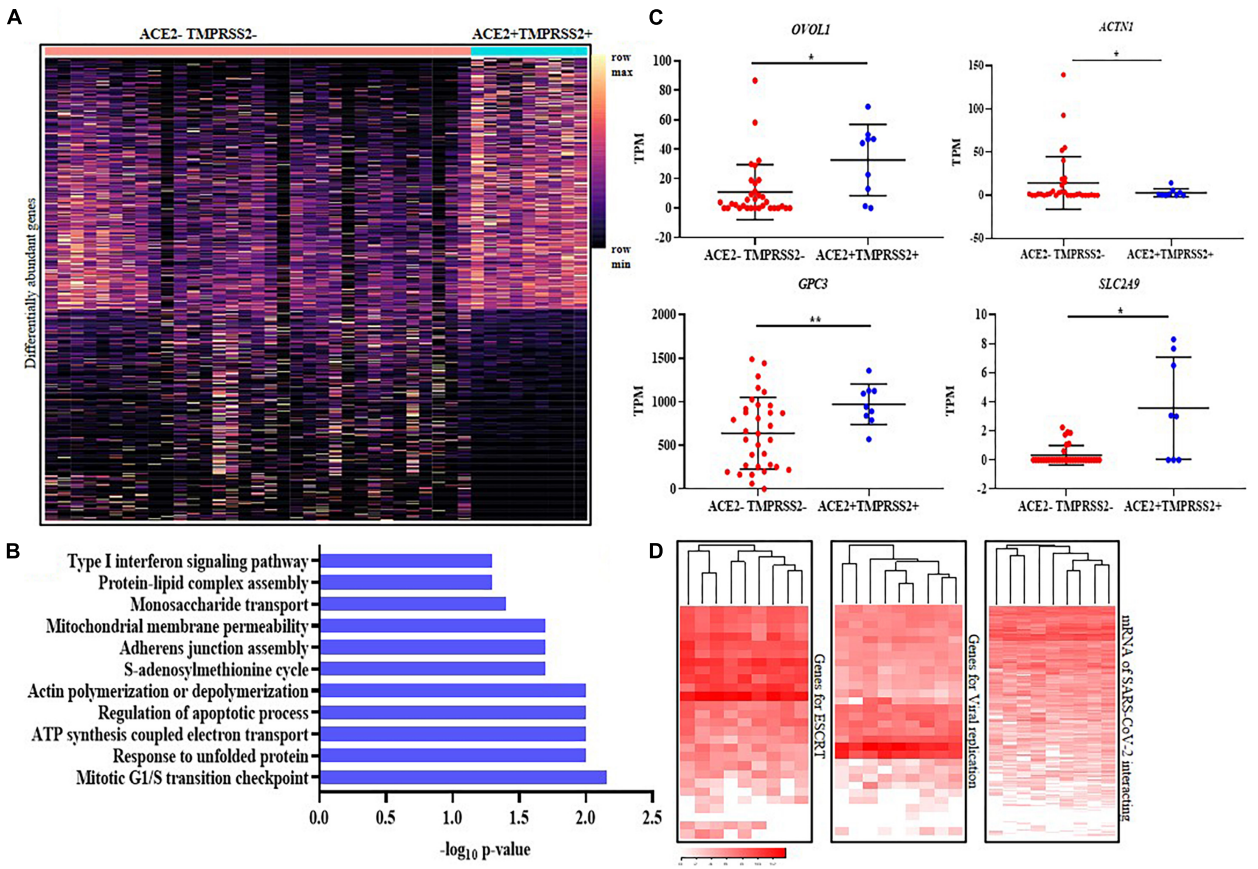


FIGURE 4 | *ACE2 + TMPRSS2 +* first trimester syncytiotrophoblast cells are terminally differentiated and express the machinery for viral endocytosis, replication and budding **(A)** Distribution of 817 differentially expressed genes in *ACE2-* and *TMPRSS2*-positive (*n* = 9) (*ACE2 + TMPRSS2 +*) and *ACE2-* and *TMPRSS2*-negative (*ACE2– TMPRSS2–*) cells (*n* = 33) of the first trimester syncytiotrophoblast. Rows represent genes and columns represent individual cells, presented on a relative color scale. **(B)** Biological processes enriched in the differentially expressed genes of the first trimester syncytiotrophoblast. The *Y* axis indicates the enriched biological processes and *X* axis is the -$\log_{10}$ of the raw *p*-values. **(C)** mRNA levels of *OVOL1*, *GPC3, SLC2A9*, and *ACTN1* in *ACE2–TMPRSS2–* and *ACE2 + TMPRSS2 +* cells. Each dot represents data of a single cell, the *Y* axis represents Transcripts Per Million (TPM). Bars denote mean ± SD. Horizontal black bars denote significantly different values (* indicates *p*-value ≤ 0.05, ** indicates *p*-value ≤ 0.001). **(D)** Heatmap showing the expression of genes involved in endosomal sorting complexes required for transport (ESCRT), replication and host genes involved in SARS-CoV-2 interaction. In all heat maps, each row depicts a gene and each column depicts a single *ACE2-* and *TMPRSS2*-positive (*n* = 9) (*ACE2 + TMPRSS2 +*) cell in the first trimester syncytiotrophoblast. The data is presented on a relative color scale in which the minimum and maximum values in each row are used to convert values to colors. Data was extracted from single cell RNA-seq of human placenta (Liu et al., 2018) [Accession number GSE89497].

these genes were expressed in most *ACE2 + TMPRSS2 +* second trimester EVTs (**Figure 5D**), while the first trimester EVTs had heterogeneous expression (**Supplementary Figure 5**).

Unique Signatures of Genes Involved in Viral Response in First Trimester Syncytiotrophoblast Cells and Second Trimester Extravillous Trophoblasts

We studied the baseline expression of 487 genes involved in viral response in both first trimester STBs and second trimester EVTs (**Supplementary Table 1**). Only a subset of viral response genes were expressed in *ACE2 + TMPRSS2 +* EVTs and STBs (**Figure 6A**). The heatmaps showed minimal heterogeneity across cells but high variability in expression across genes involved in the viral response. To characterize these genes in EVT and STB cells, genes were clustered by a hierarchical clustering method using the "hclust" function available in R. Genes were grouped based on the most optimum threshold, which resulted in four different gene clusters (**Figure 6B**). The average of the gene expression in Clusters 1 and 2 were identical in both EVTs and STBs. However, average expression of genes in Clusters 3 and 4 was more abundant in EVTs as compared to STBs (**Figure 6C**).

Next, GO analysis was performed using the PANTHER database for all four gene clusters. For each cluster, an over-representation test was performed using reference genes of PANTHER pathways, and a pathway with the highest fold-enrichment value was selected as the enriched pathway for a given gene cluster. GO classification of these clusters revealed that most of the genes in Cluster 1 had a role in the Toll-like receptor (TLR) signaling response and the genes in Cluster 2 had a role in apoptosis. Additionally, Cluster 3, which included genes of the

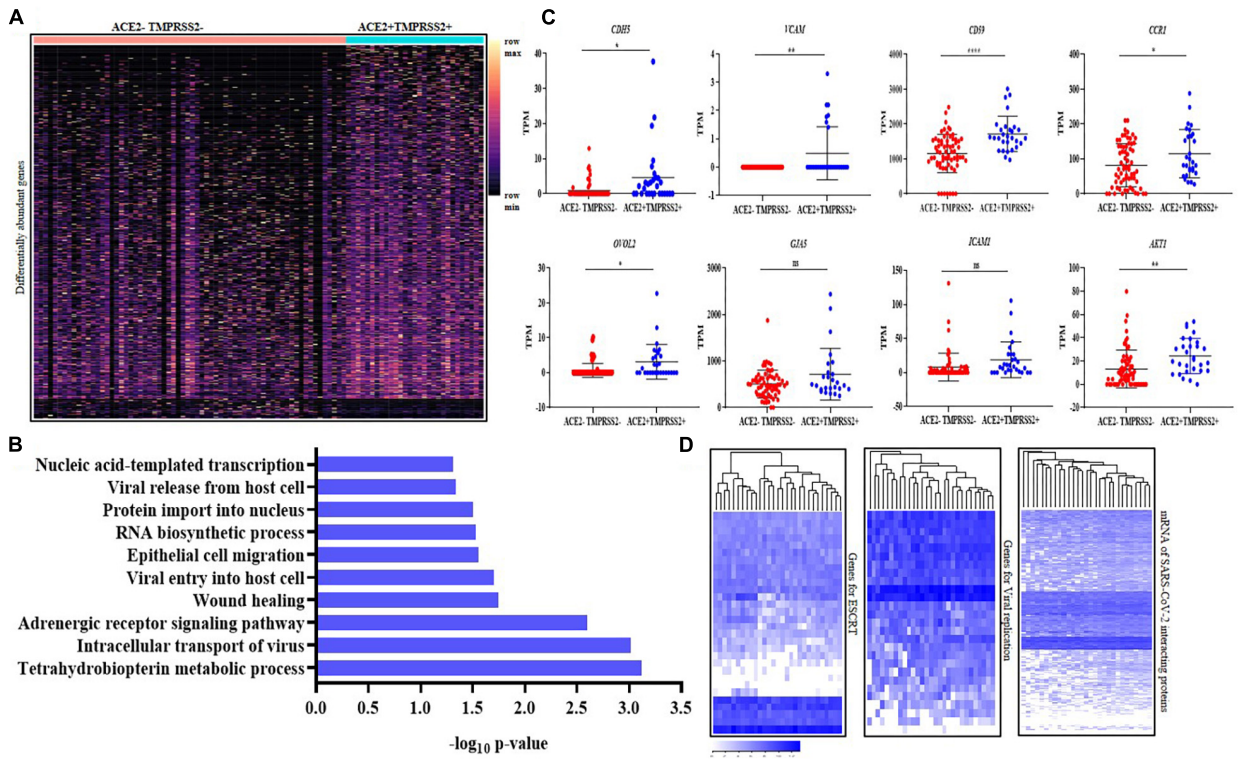


FIGURE 5 | Second trimester *ACE2* + *TMPRSS2* + cells are invasive extravillous trophoblasts and express markers of endovascular trophoblasts. **(A)** Distribution of 983 differentially expressed genes in *ACE2*- and *TMPRSS2*-positive (*n* = 29) (*ACE2* + *TMPRSS2* +) and *ACE2*- and *TMPRSS2*-negative (*n* = 66) (*ACE2–TMPRSS2–*) cells of the second trimester extravillous trophoblast. Rows represent genes and columns represent individual cells, presented on a relative color scale. **(B)** Biological processes enriched in the *ACE2* + *TMPRSS2* + cells (*n* = 29) of the second trimester extravillous trophoblast. The *Y* axis indicates the enriched biological processes and *X* axis is the enrichment score. **(C)** Comparison of mRNA level of *CDH5, VCAM, CCR1, CD59, OVOL2, ICAM*, and *AKT1* in *ACE2–TMPRSS2-* (*n* = 66) and *ACE2* + *TMPRSS2* + (*n* = 29) cells. Each dot represents data of a single cell, the *Y* axis represents Transcripts Per Million (TPM). Bars denote mean ± SD. Horizontal black bars denote significantly different values (* indicates *p*-value ≤ 0.05, ** indicates *p*-value ≤ 0.001, **** indicates *p*-value < 0.0001). **(D)** Heatmap showing the expression of genes involved in endosomal sorting complexes required for transport (ESCRT), replication and host genes involved in SARS-CoV-2 interaction. In all heat maps, each row depicts a gene and each column depicts a single *ACE2* + *TMPRSS2* + cell of the second trimester extravillous trophoblast. The data is presented on a relative color scale in which the minimum and maximum values in each row are used to convert values to colors. Data was extracted from single cell RNA-seq of human placenta (Liu et al., 2018) [Accession number GSE89497].

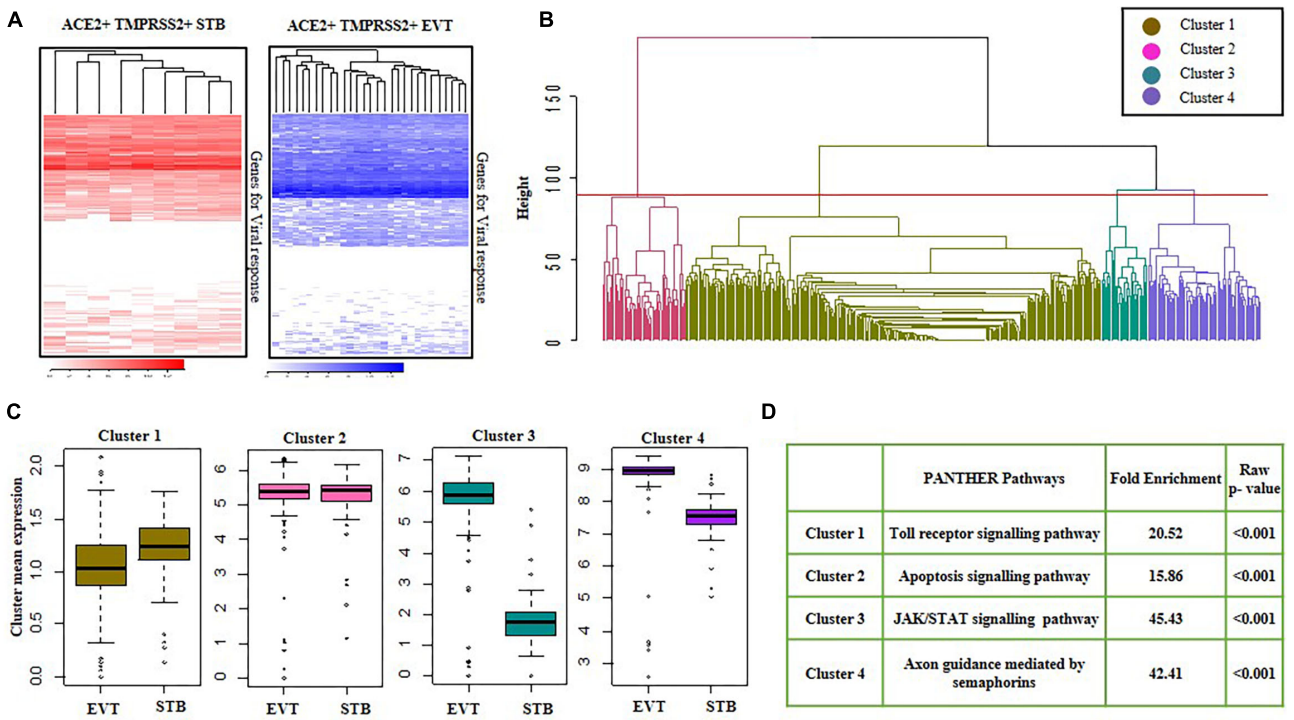
JAK-STAT pathway, and Cluster 4, which included genes for axon guidance mediated by semaphorins, were enriched in EVTs as compared to STBs (*p*-value ≤ 0.05) (**Figure 6D**).

SARS-CoV-2 Infects the Human Placenta and Is Localized in Syncytiotrophoblast Cells

To determine if SARS-CoV-2 can infect human placenta, a systematic review was carried out (**Supplementary Table 6**). Seventeen studies that reported analysis of SARS-CoV-2 in placental tissue from 93 pregnant women with COVID-19 were identified. In these studies, SARS-CoV-2 was detected by reverse transcriptase PCR (RT-PCR), immunohistochemistry or electron microscopy. Of the 93 placentae, 5 were second trimester placentae and 88 were term/preterm placentae. In all, 12% of placentae were reported to be positive for SARS-CoV-2. Second trimester placentae from all 5 women with COVID-19 were positive for SARS-CoV-2 while term/preterm placenta from ∼7% of women with COVID-19 had SARS-CoV-2 positivity. Immunohistochemistry of 1 second trimester and 2 term placentae revealed the presence of SARS-CoV-2 protein in STBs. Viral particles were also identified in STBs of second trimester and preterm (28 weeks) placenta by electron microscopy (**Supplementary Table 6**).

DISCUSSION

Herein, we utilized scRNA-seq to identify and characterize the potential cellular targets of SARS-CoV-2 infection in human placenta. To review the data presented: (1) The mRNA for coronavirus receptors (*ACE2, BSG, DPP4*, and *ANPEP*) and the S protein proteases (*TMPRSS2* and *CTSL*) are expressed in the first trimester to term placenta, (2) The SARS-CoV-2 binding receptor *ACE2* and the S protein priming protease *TMPRSS2* are co-expressed by a subset of syncytiotrophoblasts (STB) in the first trimester and extravillous trophoblasts (EVT) in the second trimester human placenta, (3) These *ACE2* + *TMPRSS2* + subsets



	PANTHER Pathways	Fold Enrichment	Raw p- value
Cluster 1	Toll receptor signalling pathway	20.52	<0.001
Cluster 2	Apoptosis signalling pathway	15.86	<0.001
Cluster 3	JAK/STAT signalling pathway	45.43	<0.001
Cluster 4	Axon guidance mediated by semaphorins	42.41	<0.001

FIGURE 6 | Viral response genes in first trimester syncytiotrophoblasts and second trimester extravillous trophoblasts. **(A)** Heatmap showing the expression of genes involved in viral response in *ACE2*- and *TMPRSS2*-positive (*ACE2* + *TMPRSS2* +) cells of first trimester STB (n = 9) and second trimester EVT (n = 29). Each row is a gene and each column represents data for a single *ACE2* and *TMPRSS2* co-expressing cell. **(B)** Cluster dendrogram of first trimester STB and second trimester EVT cells. The horizontal axis of the dendrogram represents the distance or dissimilarity between clusters. The vertical axis represents the clusters. **(C)** Mean of four clusters of upregulated genes in first trimester STB and second trimester EVT cells. *Y* axis represents cluster mean expression and *X* axis represents different cell types. **(D)** Pathways enriched in the in four clusters of first trimester STBs and second trimester EVTs, with their enrichment score and *p*-values. EVT, extravillous trophoblast; STB, syncytiotrophoblast. Data was extracted from single cell RNA-seq of human placenta (Liu et al., 2018) [Accession number GSE89497].

are highly differentiated STBs and endovascular EVTs, (4) The *ACE2* + *TMPRSS2* + placental subsets readily express mRNA for proteins involved in ESCRT of viruses and replication, in addition to transcripts for proteins that are known to interact with SARS-CoV-2 structural and non-structural proteins, and (5) The STBs and EVTs differentially express genes involved in the host response to viral infection.

Using a scRNA-seq dataset (Vento-Tormo et al., 2018), *ACE2*-positive CTBs and STBs are reported in the first trimester human placenta (Li M. et al., 2020; Singh et al., 2020). Corroborating these findings using a different dataset (Liu et al., 2018), we show the presence of *ACE2* in CTBs and STBs of first trimester placenta. However, along with STBs and CTBs, we also identified *ACE2* expression in EVTs of the first and second trimester placenta, which has not been reported earlier. In addition to *ACE2*-expressing STBs and EVTs, our study revealed that *BSG/CD147*, the alternate receptor for SARS-CoV-2 (Wang et al., 2020), is expressed by almost all the placental cells. We also detected abundant expression of *DPP4* (the receptor for MERS-CoV) and *ANPEP* (the receptor for CoV-229E) in the cells of the placenta. Like *ACE2*, *DPP4* was detected in all the cell types of the first trimester placenta and also in EVTs of the second trimester; very few STBs expressed *ANPEP*. Similar observations are made using different datasets of scRNA-seq of first trimester human placenta (Pique-Regi et al., 2020; Singh et al., 2020). In addition to the first and second trimester placenta, *ACE2, TMPRSS2, BSG, ANPEP*, and *DPP4* transcripts are also detected in human term placenta by bulk RNA-seq. However, we cannot comment on the cell types that express these genes due to the lack of publicly available scRNA-seq datasets of term human placenta. Interestingly, these genes are also expressed in the term placenta of different species including mice, cows, dogs, armadillos, elephants, opossums and bonobos. Thus, we propose that multiple cell types in the placental tissue could be targets of different coronaviruses throughout gestation.

While *ACE2* is the primary receptor for SARS-CoV-2 entry, the S protein of SARS-CoV-2 undergoes cleavage by a cell surface protease, *TMPRSS2* (Hoffmann et al., 2020). Whether *ACE2* and *TMPRSS2* are required on the same cell to activate SARS-CoV-2 S protein to invade *ACE2* single-positive cells is a matter of investigation. However, as active S protein has a finite lifetime (Shulla et al., 2011), its processing at the plasma membrane will make it most effective for viral entry. Thus, we assumed that for SARS-CoV-2, the *ACE2* and *TMPRSS2* co-expressing cells would have the highest infectivity. Our analysis revealed that a proportion of STBs (14%) in the first trimester and a subset of EVTs (15%) in second trimester human placenta co-express *ACE2* and *TMPRSS2*. Contradicting this proposition,

Pique-Regi et al. (2020) reported that co-expression of *ACE2* and *TMPRSS2* is negligible in the trophoblasts of human first, second and third trimester placenta. Differences in the methods of tissue sampling, cell isolation and inefficiencies in detection of low-abundance transcripts in scRNA-seq can underestimate the actual frequencies of *ACE2* + cells in a given tissue. Indeed, the dataset used in this study is exclusively of MACS enriched trophoblast preparations while the datasets used by Pique-Regi et al. (2019, 2020) are a mixed population of cells from the feto-maternal interface. It is known that fractionated cell preparations allow better identification of low abundance transcripts in rare cell populations (Nguyen et al., 2018). These factors could be the possible reasons that our analysis could identify more numbers of *ACE2* and *TMPRSS2* co-expressing cell types in the human placenta. While the numbers of placental cells co-expressing *ACE2* and *TMPRSS2* may appear insignificant considering the total placental volume, it must be borne in mind that only 3–6% of lung airway epithelial cell subtypes (the primary site of SARS-CoV-2 action) co-express both *ACE2* and *TMPRSS2* (Ziegler et al., 2020).

Beyond the canonical *ACE2* and *TMPRSS2* based entry, SARS-CoV-2 also utilizes *BSG*/*CD147* as the non-canonical mode of entry (Wang et al., 2020). Presently, the mechanism by which *BSG*/*CD147* mediates viral entry in host cells is unknown. In other cells, *BSG*/*CD147* promotes entry of viruses by endocytosis (Pushkarsky et al., 2001). It is possible that the same mechanism may be operative in the case of SARS-CoV-2. In this context, it is interesting that all the *BSG/CD147*-positive cells abundantly co-expressed the endosomal protease *CTSL*. Further, we observed that almost all the *ACE2* + STBs and EVTs co-expressed *BSG/CD147*, suggesting that more than one mechanism may operate for viral entry in these cells of the human placenta. Beyond *TMPRSS2*, studies have identified that SARS-CoV-2 may have a *FURIN* cleavage site, leading to a broader set of host proteases that could mediate S protein priming (Coutard et al., 2020). The *ACE2* + *TMPRSS2* + STBs and EVTs abundantly express *FURIN* as well as another endosomal protease, *CTSB*. Together our data conclusively show that multiple cells of human placenta are targets for SARS-CoV-2 binding and entry with S protein priming by both canonical and non-canonical pathways.

We next aimed to characterize the placental cells that are potential targets for SARS-CoV-2 infection. As the *ACE2*-mediated viral entry is a well-established mechanism, we focused only on characterizing the STBs of the first trimester and EVTs of the second trimester placenta that co-express both *ACE2* and *TMPRSS2* in a proportion of cells while others are devoid of these transcripts. In the developing placenta, trophoblast stem cells differentiate into cytotrophoblasts, which undergo further differentiation to form the non-self-renewing cytotrophoblasts, extravillous trophoblasts and syncytiotrophoblasts (Turco and Moffett, 2019; Hemberger et al., 2020). The syncytiotrophoblasts covering the villi are major hormone secreting cells and function as a protective immunological barrier (Maltepe and Fisher, 2015; Gupta et al., 2016; Liu et al., 2018; Vento-Tormo et al., 2018; Turco and Moffett, 2019). We observed that STBs that co-express both *ACE2* and *TMPRSS2* also express the mRNA for the peptide hormones and enzymes for steroid hormone biosynthesis. However, their levels are not significantly different from their *ACE2–TMPRSS2–* counterparts, suggesting that both these cell types retain the basic functions of STBs. However, pseudo-bulk analysis revealed that the *ACE2* + *TMPRSS2* + cells are enriched for genes involved in cell cycle checkpoints, actin filament remodeling, mitochondrial functions, hexose transport and type I interferon signaling. Indeed, the terminally differentiated STBs have replicative senescence and require extensive cytoskeletal remodeling for syncytialization; the mitochondria of STBs play a key role in progesterone synthesis by providing cholesterol (Martinez et al., 2015). Additionally, these cells are enriched in *OVOL1*, the transcription factor required for STB specification, as well as proteins involved in glucose transport across the feto-maternal barrier, a key function of well differentiated STBs (Jansson and Ylve, 2002; Renaud et al., 2015; Vento-Tormo et al., 2018; Turco and Moffett, 2019). These results imply that the *ACE2* + *TMPRSS2* + cells are a subset of highly differentiated STBs and these cells are potential targets for viral entry. Indeed, SARS-CoV-2 mRNA, protein and virions are detected in STBs of second trimester and term/preterm placenta from a woman with COVID-19 (Hosier et al., 2020). Additionally, increased syncytiotrophoblastic knots are observed in placenta from pregnant women with COVID-19 (Chen et al., 2020), which is suggestive of injury to the STBs in the placenta.

We next probed the second trimester EVTs, 15% of which co-express *ACE2* and *TMPRSS2*. The EVTs differentiate from cytotrophoblast stem cells and populate the tips of the placental villi to form the anchoring villi, thus defining the boundary between mother and fetus. The EVTs are central to placentation as they invade into the maternal decidua and are involved in remodeling of maternal spiral arteries, veins and lymphatic ducts (Sharma et al., 2016; Pollheimer et al., 2018). We observed that the *ACE2* + *TMPRSS2* + cells abundantly express the classical EVT marker *ITGB1* and also *HLA-G* that induces tolerogenic immune responses leading to acceptance of the semi-allogeneic fetus. Two kinds of EVTs are reported in the second trimester human placenta: the proliferative EVTs in the cell columns and the invasive endovascular or interstitial EVTs (Pollheimer et al., 2018; Turco and Moffett, 2019), and both of these have a unique transcript signature (Liu et al., 2018). Herein, we observed that while the levels of *TAC3* and other molecules associated with columnar versus invasive EVTs are not significantly different between *ACE2* + *TMPRSS2* + and *ACE2–TMPRSS2–* second trimester EVTs, the double-positive cells overexpressed key invasive EVT markers such as *OVOL2, GJA5, ICAM* and *AKT1* (Sharma et al., 2016; Bai et al., 2018; Liu et al., 2018; Jeyarajah et al., 2020), suggesting that these cells are invasive trophoblasts. We further observed that many of the *ACE2* + *TMPRSS2* + cells were enriched for genes having a role in cell migration. The EVTs can either invade the decidua (designated as interstitial EVTs) or remodel the spiral arteries (designated as endovascular EVTs). While both these EVTs are invasive in nature, they have differential expression of certain marker genes. For example, endovascular EVTs overexpress the CDH5 and VCAM, they also have higher expression of CCR1 and CD59 (Bulla et al., 2005; Cartwright and Balarajah, 2005; Liu et al., 2018; Ueda et al., 2019; Sato, 2020). Intriguingly, the

ACE2 + *TMPRSS2* + cells also overexpressed several of the key endovascular EVT markers including *CDH5, CCD5, CD59*, and *VCAM*, indicating that the *ACE2* + *TMPRSS2* + population of second trimester EVTs are potentially endovascular trophoblasts and are targets of SARS-CoV-2 infection. Indeed, pseudo-bulk analysis of the *ACE2* + *TMPRSS2* + cells and *ACE2*–*TMPRSS2*– cells revealed significant enrichment of genes with GO terms involving regulation of viral release from host cells. Most of the *ACE2* + *TMPRSS2* + endovascular EVTs abundantly expressed most genes whose protein products in the host are known to be involved in human endocytosis and budding of viruses and replication. Together, this data shows that SARS-CoV-2 may affect the invading EVTs at the feto-maternal interface in the second trimester and can result in damaged vasculature. In this context, it is important to note that the maternal endothelial cells in the decidua also express *ACE2* and *BSG*, making the maternal endothelium another entry point of SARS-CoV-2 infection at the feto-maternal interface. Any impairment in functions of these cells can cause placental damage and vertical transmission of the virus. Indeed, increased intervillous and subchorionic fibrin deposition and fetal thrombotic vasculopathy with zones of avascular fibrotic villi are observed in placenta of women infected with coronaviruses including SARS CoV-2 (Ng et al., 2006; Baud et al., 2020; Hosier et al., 2020; Mulvey et al., 2020). Together, these results indicate that the integrity of endovascular trophoblasts and the endothelial compartment of the feto-maternal interface may be compromised in women with SARS-CoV-2 infection.

Once the virus binds to its receptors on host cells and gains entry, it utilizes a plethora of host genes for its replication. Post replication, most enveloped viruses complete their life-cycle by forming vesicles that bud from the plasma membrane via the cellular ESCRT (endosomal sorting complexes required for transport) machinery. Interestingly, we observed that the *ACE2* + *TMPRSS2* + STBs and EVTs were enriched for the key genes that encode for proteins involved in ESCRT and viral replication. Using affinity-purification mass spectrometry, 332 human proteins that interact with SARS-CoV-2 have been identified, and many of these play a role in ESCRT and viral replication (Gordon et al., 2020). We observed that *ACE2* + *TMPRSS2* + STBs and EVTs abundantly expressed most of these genes. Thus SARS-CoV-2 may hijack proteins in the EVTs and STBs thereby interfering with normal placental functions. In this context, it is important to highlight that a significant proportion of the human SARS-CoV-2 interacting proteins also interact with proteins of other viruses including Zika and Hepatitis C virus, which replicate in the trophoblast cells (Giugliano et al., 2015; Tabata et al., 2016, 2018; Gordon et al., 2020). Together our data strongly implies that first trimester STBs and second trimester EVTs are not just targets for SARS-CoV-2 entry, but also the virus may be potentially pathogenic to these cells.

A proportion of SARS-CoV-2 proteins target the components of innate immune signaling pathways, including NF-kappa-B (Gordon et al., 2020). We decided to probe this in detail by profiling the STBs and EVTs for 487 genes whose protein products are involved in viral response in host cells. We observe that only a proportion of these genes are expressed in most EVTs and STBs. Based on their expression levels in the host cells, they could be classified in four clusters, and interestingly, the first trimester STBs and the second trimester EVTs expressed genes in the TLR signaling pathway, the primary response to viral infection. Previous studies in SARS-CoV have identified involvement of TLR pathways in protection against viral response (Dosch et al., 2009; Totura et al., 2015). However, we found that the genes in the JAK-STAT pathway were overexpressed in the EVTs but not in the STBs. However, this is not surprising as the JAK-STAT pathway is required for physiological functions of EVTs, namely invasion (Fitzgerald et al., 2010; Suman et al., 2013; Sharma et al., 2016; Godbole et al., 2017). The JAK-STAT pathway is also central for mounting a host response to viral infection, and treatment with interferon gamma induces the expression of interferon-stimulated genes in EVT cells (Verma et al., 2020). Thus, EVTs are not only the entry sites for SARS-CoV-2 infection, but they also possess the cellular machinery to mount an inflammatory response toward an infection. With regard to coronaviruses (including SARS-CoV-2), an overexuberant inflammatory response is observed even at lower viral titres, which contributes to the viral pathogenicity in the lung (Liao et al., 2020). Further, the *ACE2* receptors are induced by interferon signaling in the lung (Ziegler et al., 2020), thereby amplifying the infectious cycle in host tissues. Whether or not a similar mechanism is operative in placental cells is under investigation, but the heightened baseline expression of the JAK-STAT pathway genes in the EVTs itself could readily lead to placental inflammation that may be detrimental to pregnancy. Infiltration of leukocytes and chorioamnionitis is observed in placenta from women with coronavirus infection (Ng et al., 2006; Baud et al., 2020). Since inflammation of the feto-maternal interface causes preterm births (Silasi et al., 2015; Surve et al., 2016), it is plausible that the increased incidence of preterm delivery in women with COVID-19 could be linked to this process.

Beyond preterm births, the demonstration that the *ACE2* + *TMPRSS2* + subpopulation of EVTs consists of invasive endovascular trophoblasts is clinically relevant in conditions like preeclampsia. The invasion of the trophoblast cells and remodeling of the spiral arteries deep into the myometrium is essential for normal fetal growth and development (Norwitz, 2006; Soares et al., 2015). If the arteries are not sufficiently remodeled, there is disordered perfusion of blood and an inadequate supply of nutrients and oxygen, resulting in fetal growth restriction, stillbirth, preeclampsia, placental abruption and preterm labor (Brosens et al., 2019). Since SARS-CoV-2 and other coronaviruses may target the endovascular trophoblasts, it is plausible that the infection could lead to other adverse pregnancy outcomes. Indeed, higher incidence of preeclampsia, preterm labor, fetal distress and premature rupture of membranes are reported in pregnant women infected with SARS-CoV-2 in the third trimester (Gajbhiye et al., 2020). Also, high rates of miscarriages, preterm birth and premature rupture of membranes have been reported for other human coronavirus infections (Alfaraj et al., 2019; Mullins et al., 2020). These observations imply that SARS-CoV-2 infection is detrimental to pregnancy due the possible infection of placental cells such as EVTs.

To determine whether SARS-CoV-2 can infect the placental cells, we carried out a systematic review to identify studies that report presence or absence of the virus in placenta of women with COVID-19. The results revealed that ∼12% (11/93) of placentae obtained from mothers with COVID-19 had detectable levels of SARS-CoV-2 RNA (**Supplementary Table 6**). Viral RNA, non-structural proteins and intact virions are detected in STBs of the second trimester and preterm/term placenta. This is definitive evidence of placental infection by SARS-CoV-2. However, this may not be an accurate estimate of the frequency of placental infection as most studies included women at term with unknown viral loads and duration of infection. Nevertheless, the fact that some studies have also detected the virus in the amniotic fluid and fetal membranes (Baud et al., 2020; Zamaniyan et al., 2020) as well as IgM in fetal blood (Zeng et al., 2020) implies that the placenta does get infected and the virus can cross the transplacental barrier to infect the fetus. In a systematic review and primary data from a large cohort of pregnant women, mother-to-child transmission of the virus is observed in 5–8% of cases (Gajbhiye et al., 2020; Knight et al., 2020). Thus, it appears likely that the placenta is not just permissive to viral entry but can be a site of active viremia that can lead to the breakthrough of SARS-CoV-2 infection from mother to fetus.

To summarize, this is the first in-depth survey to identify the cellular basis of SARS-CoV-2 infection in the human placenta. However, this data is limited by the constraints of scRNA-seq which includes host and environmental factors that may affect the expression of receptors and proteases. Experimental variations like sites of tissue collection, cell isolation techniques and statistical cut-offs may lead to inclusion or exclusion of specific cell types. Furthermore, our observations need to be corroborated for protein expression. Nevertheless, our results provide a basic framework in understanding of the paraphernalia involved in SARS-CoV-2 infections in pregnancy. It will be essential to determine how SARS-CoV-2 infection alters the temporal dynamics of host responses at the single-cell resolution in the placenta. We believe that this work will aid in developing rational strategies for management of COVID-19 and other coronavirus infections in pregnancy.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

NA, AB, and PC analyzed and interpreted the data and prepared the figures. SC and AM were involved in data analysis and preparing the manuscript. KC prepared the figures and edited the manuscript. DM conceived the idea and planned this study. DM and MJ spearheaded the study and were involved in data interpretation. All authors were involved in manuscript writing and approved the final version.

FUNDING

DM lab was funded by grants from Indian Council of Medical Research (ICMR), Government of India and IMPRINT IIC grant (IMP/2019/000115/HT) from Department of Science and Technology, Government of India. MJ was supported by Ramanujan Fellowship (SB/S2/RJN-049/2018) awarded by SERB, DST, Government of India. SC was supported by funding from Department of Health Research, Government of India.

ACKNOWLEDGMENTS

We are grateful to Mr. Domdatt Singh for help in editing the manuscript. The study makes use of the datasets bearing the accession numbers GSE89497, GSE124282, and GSE79121. We express our gratitude to the authors for making these datasets publically available. KC is thankful to Nehru-Fulbright Scholarship. AM is thankful to University Grants Commission (UGC) Government of India for research fellowship. NA and AB are thankful to DST-SERB for project assistantship. The manuscript bears the NIRRH ID RA/908/05-2020. The pre-print of this manuscript is available at https://www.preprints.org/manuscript/202005.0195/v1.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.00783/full#supplementary-material

REFERENCES

Ahmed, I., Akram, Z., Iqbal, H. M. N., and Munn, A. L. (2019). The regulation of endosomal sorting complex required for transport and accessory proteins in multivesicular body sorting and enveloped viral budding - An overview. *Int. J. Biol. Macromol.* 127, 1–11. doi: 10.1016/j.ijbiomac.2019.01.015

Alfaraj, S. H., Al-Tawfiq, J. A., and Memish, Z. A. (2019). Middle East respiratory syndrome coronavirus (MERS-CoV) infection during pregnancy: report of two cases & review of the literature. *J. Microbiol. Immunol. Infect.* 52, 501–503. doi: 10.1016/j.jmii.2018.04.005

Armstrong, D. L., McGowen, M. R., Weckle, A., Pantham, P., Caravas, J., Agnew, D., et al. (2017). The core transcriptome of mammalian placentas and the

divergence of expression with placental shape. *Placenta* 57, 71–78. doi: 10.1016/j.placenta.2017.04.015

Arora, N., Sadovsky, Y., Dermody, T. S., and Coyne, C. B. (2018). Microbial vertical transmission during human pregnancy. *Cell* 21, 561–567. doi: 10.1016/j.chom.2017.04.007.Microbial

Ashray, N., Bhide, A., Chakarborty, P., Colaco, S., Mishra, A., Chhabria, K., et al. (2020). Single-cell RNA-seq identifies cell subsets in human placenta that highly expresses factors to drive pathogenesis of SARS-CoV-2. *Preprints* 2020050195. doi: 10.20944/preprints202005.0195.v1

Bai, R., Kusama, K., Nakamura, K., Sakurai, T., Kimura, K., Ideta, A., et al. (2018). Down-regulation of transcription factor OVOL2 contributes to epithelial-mesenchymal transition in a noninvasive type of trophoblast implantation to the maternal endometrium. *FASEB J.* 32, 3371–3384. doi: 10.1096/fj.201701131RR

Baud, D., Greub, G., Frave, G., Gengler, C., Jaton, K., Dubruc, E., et al. (2020). Second-Trimester Miscarriage in a Pregnant Woman With SARS-CoV-2 Infection. *JAMA J. Am. Med. Assoc.* 323, 2198–2200. doi: 10.1001/jama.2020.7233

Bharadwaj, M. S., Strawn, W. B., Groban, L., Yamaleyeva, L. M., Chappell, M. C., Horta, C., et al. (2011). Angiotensin-converting enzyme 2 deficiency is associated with impaired gestational weight gain and fetal growth restriction. *Hypertension* 58, 852–858. doi: 10.1161/HYPERTENSIONAHA.111.179358

Bharti, S., Narad, P., Chugh, P., Choudhury, A., Bhatnagar, S., and Sengupta, A. (2020). Multi-parametric disease dynamics study and analysis of the COVID-19 epidemic and implementation of population-wide intrusions: the Indian perspective. *medRxiv* [Preprint]. doi: 10.1101/2020.06.02.20120360

Brosens, I., Puttemans, P., and Benagiano, G. (2019). Placental bed research: I. The placental bed: from spiral arteries remodeling to the great obstetrical syndromes. *Am. J. Obstet. Gynecol.* 221, 437–456. doi: 10.1016/j.ajog.2019.05.044

Bulla, R., Villa, A., Bossi, F., Cassetti, A., Radillo, O., Spessotto, P., et al. (2005). VE-cadherin is a critical molecule for trophoblast-endothelial cell interaction in decidual spiral arteries. *Exp. Cell Res.* 303, 101–113. doi: 10.1016/j.yexcr.2004.09.015

Burton, G. J., Fowden, A. L., and Thornburg, K. L. (2016). Placental origins of chronic disease. *Physiol. Rev.* 96, 1509–1565. doi: 10.1152/physrev.00029.2015

Cartwright, J. E., and Balarajah, G. (2005). Trophoblast interactions with endothelial cells are increased by interleukin-1β and tumour necrosis factor α and involve vascular cell adhesion molecule-1 and α4β1. *Exp. Cell Res.* 304, 328–336. doi: 10.1016/j.yexcr.2004.11.013

Chen, S., Huang, B., Luo, D. J., Li, X., Yang, F., Zhao, Y., et al. (2020). [Pregnant women with new coronavirus infection: a clinical characteristics and placental pathological analysis of three cases]. *Chinese J. Pathol.* 49:E005. doi: 10.3760/cma.j.cn112151-20200225-00138

Colaco, S., Chhabria, K., Singh, N., Bhide, A., Singh, D., Singh, A., et al. (2020). *Expression of SARS-CoV-2 Receptor ACE2 and the Spike Protein Processing Enzymes in Developing Human Embryos*. Available online at: http://arxiv.org/abs/2004.04935 (accessed May 7, 2020).

Coutard, B., Valle, C., de Lamballerie, X., Canard, B., Seidah, N. G., and Decroly, E. (2020). The spike glycoprotein of the new coronavirus 2019-nCoV contains a furin-like cleavage site absent in CoV of the same clade. *Antiviral Res.* 176:104742. doi: 10.1016/j.antiviral.2020.104742

de Wilde, A. H., Snijder, E. J., Kikkert, M., and van Hemert, M. J. (2018). Host factors in coronavirus replication. *Curr. Top. Microbiol. Immunol.* 49, 1–42. doi: 10.1007/82_2017_25

Deshpande, S. S., and Balasinor, N. H. (2018). Placental defects: an epigenetic perspective. *Reprod. Sci.* 25, 1143–1160. doi: 10.1177/1933719118766265

Dosch, S. F., Mahajan, S. D., and Collins, A. R. (2009). SARS coronavirus spike protein-induced innate immune response occurs via activation of the NF-κB pathway in human monocyte macrophages in vitro. *Virus Res.* 142, 19–27. doi: 10.1016/j.virusres.2009.01.005

Fitzgerald, J. S., Germeyer, A., Huppertz, B., Jeschke, U., Knöfler, M., Moser, G., et al. (2010). Governing the invasive trophoblast: current aspects on intra- and extracellular regulation. *Am. J. Reprod. Immunol.* 63, 492–505. doi: 10.1111/j.1600-0897.2010.00824.x

Gajbhiye, R., Modi, D., and Mahale, S. (2020). Pregnancy outcomes, newborn complications and maternal-fetal transmission of SARS-CoV-2 in women with COVID-19: a systematic review. *medRxiv* [Preprint]. doi: 10.1101/2020.04.11.20062356

Galinato, M., Shimoda, K., Aguiar, A., Hennig, F., Boffelli, D., McVoy, M. A., et al. (2019). Single-cell transcriptome analysis of CD34+stem cell-derived myeloid cells infected with human cytomegalovirus. *Front. Microbiol.* 10:577. doi: 10.3389/fmicb.2019.00577

Gatta, A. T., and Carlton, J. G. (2019). The ESCRT-machinery: closing holes and expanding roles. *Curr. Opin. Cell Biol.* 59, 121–132. doi: 10.1016/j.ceb.2019.04.005

Giugliano, S., Petroff, M. G., Warren, B. D., Jasti, S., Linscheid, C., Ward, A., et al. (2015). Hepatitis C virus sensing by human trophoblasts induces innate immune responses and recruitment of maternal NK cells: potential implications for limiting vertical transmission. *J. Immunol.* 195, 3737–3747. doi: 10.4049/jimmunol.1500409

Godbole, G., Suman, P., Malik, A., Galvankar, M., Joshi, N., Fazleabas, A., et al. (2017). Decrease in expression of HOXA10 in the decidua after embryo implantation promotes trophoblast invasion. *Endocrinology* 158, 2618–2633. doi: 10.1210/en.2017-00032

Gordon, D. E., Jang, G. M., Bouhaddou, M., Xu, J., Obernier, K., White, K. M., et al. (2020). A SARS-CoV-2 protein interaction map reveals targets for drug repurposing. *Nature* 583, 459–468. doi: 10.1038/s41586-020-2286-9

Gupta, N., Praharaj, I., Bhatnagar, T., Thangaraj, J. W. V., Giri, S., Chauhan, H., et al. (2020). Severe acute respiratory illness surveillance for coronavirus disease 2019, India, 2020. *Indian J. Med. Res.* 151, 236–240. doi: 10.4103/ijmr.IJMR_1035_20

Gupta, S. K., Malhotra, S. S., Malik, A., Verma, S., and Chaudhary, P. (2016). Cell signaling pathways involved during invasion and syncytialization of trophoblast cells. *Am. J. Reprod. Immunol.* 75, 361–371. doi: 10.1111/aji.12436

Hemberger, M., Hanna, C. W., and Dean, W. (2020). Mechanisms of early placental development in mouse and humans. *Nat. Rev. Genet.* 21, 27–43. doi: 10.1038/s41576-019-0169-4

Hirsch, A. J., Roberts, V. H. J., Grigsby, P. L., Haese, N., Schabel, M. C., Wang, X., et al. (2018). Zika virus infection in pregnant rhesus macaques causes placental dysfunction and immunopathology. *Nat. Commun.* 9, 1–15. doi: 10.1038/s41467-017-02499-9

Hoffmann, M., Kleine-Weber, H., Schroeder, S., Krüger, N., Herrler, T., Erichsen, S., et al. (2020). SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor. *Cell* 181, 271.e8–280.e8. doi: 10.1016/j.cell.2020.02.052

Hosier, H., Farhadian, S. F., Morotti, R. A., Deshmukh, U., Lu-Culligan, A., Campbell, K. H., et al. (2020). SARS-CoV-2 infection of the placenta. *J. Clin. Invest.* (in press). doi: 10.1172/JCI139569

Iyer, A., Gupta, K., Sharma, S., Hari, K., Lee, Y. F., Ramalingam, N. N., et al. (2020). Integrative analysis and machine learning based characterization of single circulating tumor cells. *J. Clin. Med.* 9:1206. doi: 10.3390/jcm9041206

Jagtap, D., Selvaa Kumar, C., Mahale, S., and Patel, V. (2020). Modelling and docking of Indian SARS-CoV-2 spike protein 1 with ACE2: implications for co-morbidity and therapeutic intervention. *arXiv* [Preprint]. Available online at: http://arxiv.org/abs/2004.06361 (accessed June 12, 2020).

Jansson, T., and Ylve, K. (2002). Glucose transport and system a activity in syncytiotrophoblast microvillous and basal plasma membranes in intrauterine growth. *Placenta* 23, 392–399. doi: 10.1053/plac.2002.0826

Jeyarajah, M. J., Jaju Bhattad, G., Hillier, D. M., and Renaud, S. J. (2020). The transcription factor OVOL2 represses ID2 and drives differentiation of trophoblast stem cells and placental development in mice. *Cells* 9:840. doi: 10.3390/cells9040840

Knight, M., Bunch, K., Vousden, N., Morris, E., Simpson, N., Gale, C., et al. (2020). Characteristics and outcomes of pregnant women admitted to hospital with confirmed SARS-CoV-2 infection in UK: national population based cohort study. *BMJ* 369:m2107. doi: 10.1136/bmj.m2107

Lee, C. L., Lam, M. P. Y., Lam, K. K. W., Leung, C. O. N., Pang, R. T. K., Chu, I. K., et al. (2013). Identification of CD147 (basigin) as a mediator of trophoblast functions. *Hum. Reprod.* 28, 2920–2929. doi: 10.1093/humrep/det355

Letko, M., Marzi, A., and Munster, V. (2020). Functional assessment of cell entry and receptor usage for SARS-CoV-2 and other lineage B betacoronaviruses. *Nat. Microbiol.* 5, 562–569. doi: 10.1038/s41564-020-0688-y

Li, M., Chen, L., Zhang, J., Xiong, C., and Li, X. (2020). The SARS-CoV-2 receptor ACE2 expression of maternal-fetal interface and fetal organs by single-cell transcriptome study. *PLoS One* 15:e0230295. doi: 10.1371/journal.pone.0230295

Li, R., Pei, S., Chen, B., Song, Y., Zhang, T., Yang, W., et al. (2020). Substantial undocumented infection facilitates the rapid dissemination of novel coronavirus (SARS-CoV2). *Science* 368, 489–493. doi: 10.1126/science.abb3221

Liao, M., Liu, Y., Yuan, J., Wen, Y., Xu, G., Zhao, J., et al. (2020). The landscape of lung bronchoalveolar immune cells in COVID-19 revealed by single-cell RNA sequencing. *medRxiv* [Preprint]. doi: 10.1101/2020.02.23.20026690

Liu, Y., Fan, X., Wang, R., Lu, X., Dang, Y. L., Wang, H. H., et al. (2018). Single-cell RNA-seq reveals the diversity of trophoblast subtypes and patterns of differentiation in the human placenta. *Cell Res.* 28, 819–832. doi: 10.1038/s41422-018-0066-y

Lukassen, S., Chua, R. L., Trefzer, T., Kahn, N. C., Schneider, M. A., Muley, T., et al. (2020). SARS-CoV-2 receptor ACE2 and TMPRSS2 are predominantly expressed in a transient secretory cell type in subsegmental bronchial branches. *bioRxiv* [preprint]. doi: 10.1101/2020.03.13.991455

Maltepe, E., and Fisher, S. J. (2015). Placenta: the forgotten organ. *Annu. Rev. Cell Dev. Biol.* 31, 523–552. doi: 10.1146/annurev-cellbio-100814-125620

Martinez, F., Olvera-Sanchez, S., Esparza-Perusquia, M., Gomez-Chang, E., and Flores-Herrera, O. (2015). Multiple functions of syncytiotrophoblast mitochondria. *Steroids* 103, 11–22. doi: 10.1016/j.steroids.2015.09.006

Mullins, E., Evans, D., Viner, R. M., O'Brien, P., and Morris, E. (2020). Coronavirus in pregnancy and delivery: rapid review. *Ultrasound Obstet. Gynecol.* 55, 586–592. doi: 10.1002/uog.22014

Mulvey, J. J., Magro, C. M., Ma, L. X., Nuovo, G. J., and Baergen, R. N. (2020). Analysis of complement deposition and viral RNA in placentas of COVID-19 patients. *Ann. Diagn. Pathol.* 46:151529. doi: 10.1016/j.anndiagpath.2020.151529

Ng, W. F., Wong, S. F., Lam, A., Mak, Y. F., Yao, H., Lee, K. C., et al. (2006). The placentas of patients with severe acute respiratory syndrome: a pathophysiological evaluation. *Pathology* 38, 210–218. doi: 10.1080/00313020600696280

Nguyen, Q. H., Pervolarakis, N., Nee, K., and Kessenbrock, K. (2018). Experimental considerations for single-cell RNA sequencing approaches. *Front. Cell Dev. Biol.* 6:108. doi: 10.3389/fcell.2018.00108

Norwitz, E. R. (2006). Defective implantation and placentation: laying the blueprint for pregnancy complications. *Reprod. Biomed. Online* 13, 591–599. doi: 10.1016/S1472-6483(10)60649-9

Pique-Regi, R., Romero, R., Tarca, A. L., Luca, F., Xu, Y., Alazizi, A., et al. (2020). Does the human placenta express the canonical cell entry mediators for SARS-CoV-2? *BioRxiv* [Preprint]. doi: 10.1101/2020.05.18.101485

Pique-Regi, R., Romero, R., Tarca, A. L., Sendler, E. D., Xu, Y., Garcia-Flores, V., et al. (2019). Single cell transcriptional signatures of the human placenta in term and preterm parturition. *eLife* 8:e52004. doi: 10.7554/eLife.52004

Pollheimer, J., Vondra, S., Baltayeva, J., Beristain, A. G., and Knöfler, M. (2018). Regulation of placental extravillous trophoblasts by the maternal uterine environment. *Front. Immunol.* 9:2597. doi: 10.3389/fimmu.2018.02597

Prajapat, M., Sarma, P., Shekhar, N., Avti, P., Sinha, S., Kaur, H., et al. (2020). Drug targets for corona virus: a systematic review. *Indian J. Pharmacol.* 52, 56–65. doi: 10.4103/ijp.IJP_115_20

Pushkarsky, T., Zybarth, G., Dubrovsky, L., Yurchenko, V., Tang, H., Guo, H., et al. (2001). CD147 facilitates HIV-1 infection by interacting with virus-associated cyclophilin A. *Proc. Natl. Acad. Sci. U.S.A.* 98, 6360–6365. doi: 10.1073/pnas.111583198

Qi, J., Zhou, Y., Hua, J., Zhang, L., Bian, J., Liu, B., et al. (2020). The scRNA-seq expression profiling of the receptor ACE2 and the cellular protease TMPRSS2 reveals human organs susceptible to COVID-19 infection. *BioRxiv* [Preprint]. doi: 10.1101/2020.04.16.045690

Renaud, S. J., Chakraborty, D., Mason, C. W., Karim Rumi, M. A., Vivian, J. L., and Soares, M. J. (2015). OVO-like 1 regulates progenitor cell fate in human trophoblast development. *Proc. Natl. Acad. Sci. U.S.A.* 112, E6175–E6184. doi: 10.1073/pnas.1507397112

Russell, A. B., Trapnell, C., and Bloom, J. D. (2018). Extreme heterogeneity of influenza virus infection in single cells. *eLife* 7, 1–26. doi: 10.7554/eLife.32303

Sato, Y. (2020). Endovascular trophoblast and spiral artery remodeling. *Mol. Cell. Endocrinol.* 503:110699. doi: 10.1016/j.mce.2019.110699

Seow, J. J. W., Pai, R., Mishra, A., Shepherdson, E., Lim, T. K. H., Goh, B. K. P., et al. (2020). scRNA-seq reveals ACE2 and TMPRSS2 expression in TROP2+ Liver Progenitor Cells: implications in COVID-19 associated Liver Dysfunction. *BioRxiv* [Preprint]. doi: 10.1101/2020.03.23.002832

Shang, J., Ye, G., Shi, K., Wan, Y., Luo, C., Aihara, H., et al. (2020). Structural basis of receptor recognition by SARS-CoV-2. *Nature* 581, 221–224. doi: 10.1038/s41586-020-2179-y

Sharma, A., Cao, E. Y., Kumar, V., Zhang, X., Leong, H. S., Wong, A. M. L., et al. (2018). Longitudinal single-cell RNA sequencing of patient-derived primary cells reveals drug-induced infidelity in stem cell hierarchy. *Nat. Commun.* 9:4931. doi: 10.1038/s41467-018-07261-3

Sharma, S., Godbole, G., and Modi, D. (2016). Decidual control of trophoblast invasion. *Am. J. Reprod. Immunol.* 75, 341–350. doi: 10.1111/aji.12466

Shulla, A., Heald-Sargent, T., Subramanya, G., Zhao, J., Perlman, S., and Gallagher, T. (2011). A transmembrane serine protease is linked to the severe acute respiratory syndrome coronavirus receptor and activates virus entry. *J. Virol.* 85, 873–882. doi: 10.1128/jvi.02062-10

Silasi, M., Cardenas, I., Kwon, J. Y., Racicot, K., Aldo, P., and Mor, G. (2015). Viral infections during pregnancy. *Am. J. Reprod. Immunol.* 73, 199–213. doi: 10.1111/aji.12355

Singh, M., Bansal, V., Feschotte, C., and Feschotte, C. (2020). A single-cell RNA expression map of human coronavirus entry factors. *bioRxiv* [Preprint]. doi: 10.1101/2020.05.08.084806

Soares, M. J., Chakraborty, D., Kubota, K., and Renaud, S. J. (2015). Adaptive mechanisms controlling uterine spiral artery remodeling during the establishment of pregnancy. *Int. J. Dev. Biol.* 58, 247–259. doi: 10.1387/ijdb.140083ms

Suman, P., Malhotra, S. S., and Gupta, S. K. (2013). LIF-STAT signaling and trophoblast biology. *JAK-STAT* 2:e25155. doi: 10.4161/jkst.25155

Sungnak, W., Huang, N., Bécavin, C., Berg, M., Queen, R., Litvinukova, M., et al. (2020). SARS-CoV-2 entry factors are highly expressed in nasal epithelial cells together with innate immune genes. *Nat. Med.* 26, 681–687. doi: 10.1038/s41591-020-0868-6

Surve, M. V., Anil, A., Kamath, K. G., Bhutda, S., Sthanam, L. K., Pradhan, A., et al. (2016). Membrane vesicles of group B *Streptococcus* disrupt feto-maternal barrier leading to preterm birth. *PLoS Pathog.* 12:e1005816. doi: 10.1371/journal.ppat.1005816

Suryawanshi, H., Morozov, P., Straus, A., Sahasrabudhe, N., Max, K. E. A., Garzia, A., et al. (2018). A single-cell survey of the human first-trimester placenta and decidua. *Sci. Adv.* 4, 1–12. doi: 10.1126/sciadv.aau4788

Szabo, P. A., Levitin, H. M., Miron, M., Snyder, M. E., Senda, T., Yuan, J., et al. (2019). Single-cell transcriptomics of human T cells reveals tissue and activation signatures in health and disease. *Nat. Commun.* 10:4706. doi: 10.1038/s41467-019-12464-3

Tabata, T., Petitt, M., Puerta-Guardo, H., Michlmayr, D., Harris, E., and Pereira, L. (2018). Zika virus replicates in proliferating cells in explants from first-trimester human placentas, potential sites for dissemination of infection. *J. Infect. Dis.* 217, 1202–1213. doi: 10.1093/infdis/jix552

Tabata, T., Petitt, M., Puerta-Guardo, H., Michlmayr, D., Wang, C., Fang-Hoover, J., et al. (2016). Zika virus targets different primary human placental cells, suggesting two routes for vertical transmission. *Cell Host Microbe* 20, 155–166. doi: 10.1016/j.chom.2016.07.002

Totura, A. L., Whitmore, A., Agnihothram, S., Schäfer, A., Katze, M. G., Heise, M. T., et al. (2015). Toll-like receptor 3 signaling via TRIF contributes to a protective innate immune response to severe acute respiratory syndrome coronavirus infection. *mBio* 6:e00638-15. doi: 10.1128/mBio.00638-15

Turco, M. Y., and Moffett, A. (2019). Development of the human placenta. *Development* 146, 1–14. doi: 10.1242/dev.163428

Ueda, M., Sato, Y., Horie, A., Tani, H., Miyazaki, Y., Okunomiya, A., et al. (2019). Endovascular trophoblast expresses CD59 to evade complement-dependent cytotoxicity. *Mol. Cell. Endocrinol.* 490, 57–67. doi: 10.1016/j.mce.2019.04.006

Vento-Tormo, R., Efremova, M., Botting, R. A., Turco, M. Y., Vento-Tormo, M., Meyer, K. B., et al. (2018). Single-cell reconstruction of the early maternal–fetal interface in humans. *Nature* 563, 347–353. doi: 10.1038/s41586-018-0698-6

Verma, S., Kang, A. K., Pal, R., and Gupta, S. K. (2020). BST2 regulates interferon gamma-dependent decrease in invasion of HTR-8/SVneo cells via STAT1 and AKT signaling pathways and expression of E-cadherin. *Cell Adhes. Migr.* 14, 24–41. doi: 10.1080/19336918.2019.1710024

Wang, B., Wang, P., Parobchak, N., Treff, N., Tao, X., Wang, J., et al. (2019). Integrated RNA-seq and ChIP-seq analysis reveals a feed-forward loop regulating H3K9ac and key labor drivers in human placenta. *Placenta* 76, 40–50. doi: 10.1016/j.placenta.2019.01.010

Wang, K., Chen, W., Zhou, Y.-S., Lian, J.-Q., Zhang, Z., Du, P., et al. (2020). SARS-CoV-2 invades host cells via a novel route: CD147-spike protein. *bioRxiv* [Preprint]. doi: 10.1101/2020.03.14.988345

Zamaniyan, M., Ebadi, A., Aghajanpoor Mir, S., Rahmani, Z., Haghshenas, M., and Azizi, S. (2020). Preterm delivery in pregnant woman with critical COVID - 19 pneumonia and vertical transmission. *Prenat. Diagn.* [Epub ahead of print]. doi: 10.1002/pd.5713

Zeng, H., Xu, C., Fan, J., Tang, Y., Deng, Q., Zhang, W., et al. (2020). Antibodies in Infants Born to Mothers with COVID-19 Pneumonia. *JAMA J. Am. Med. Assoc.* 323, 1848–1849. doi: 10.1001/jama.2020.4861

Zhang, H., Kang, Z., Gong, H., Xu, D., Wang, J., Li, Z. Z., et al. (2020). Digestive system is a potential route of COVID-19: an analysis of single-cell coexpression pattern of key proteins in viral entry process. *Gut* (in press). doi: 10.1136/gutjnl-2020-320953

Zhu, N., Zhang, D., Wang, W., Li, X., Yang, B., Song, J., et al. (2020). A novel coronavirus from patients with pneumonia in China, 2019. *N. Engl. J. Med.* 382, 727–733. doi: 10.1056/NEJMoa2001017

Ziegler, C., Allon, S. J., Nyquist, S. K., Mbano, I., Miao, V. N., Cao, Y., et al. (2020). SARS-CoV-2 Receptor ACE2 is an interferon-stimulated gene in human airway epithelial cells and is enriched in specific cell subsets across tissues. *SSRN Electron. J.* 181, 1016.e19–1035.e19. doi: 10.2139/ssrn.3555145

Zou, X., Chen, K., Zou, J., Han, P., Hao, J., and Han, Z. (2020). Single-cell RNA-seq data analysis on the receptor ACE2 expression reveals the potential risk of different human organs vulnerable to 2019-nCoV infection. *Front. Med.* 14, 185–192. doi: 10.1007/s11684-020-0754-0

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 19 August 2020
doi: 10.3389/fimmu.2020.01880

The 2020 Pandemic: Current SARS-CoV-2 Vaccine Development

Sana O. Alturki[1,2], *Sawsan O. Alturki*[1,2], *Jennifer Connors*[1,3], *Gina Cusimano*[1], *Michele A. Kutzler*[1,3], *Abdullah M. Izmirly*[1,2,3]* *and Elias K. Haddad*[1,3]*

[1] *Department of Microbiology and Immunology, Drexel University College of Medicine, Philadelphia, PA, United States,*
[2] *Department of Medical Technology, Faculty of Applied Medical Sciences, King Abdulaziz University, Jeddah, Saudi Arabia,*
[3] *Division of Infectious Diseases and HIV Medicine, Department of Medicine, Drexel University College of Medicine, Philadelphia, PA, United States*

Coronaviruses are enveloped viruses with a positive-sense single-stranded RNA genome infecting animals and humans. Coronaviruses have been described more than 70 years ago and contain many species. Severe Acute Respiratory Syndrome (SARS) and Middle East Respiratory Syndrome (MERS) are lethal species caused by human coronaviruses (HCoVs). Currently, a novel strain of HCoVs, named Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) causes coronavirus disease 2019 (Covid-19). SARS-CoV-2 was first identified in December 2019 in Wuhan, the capital city of the Hubei province of China, and has since spread worldwide causing an outbreak in more than 200 countries. The SARS-CoV-2 outbreak was declared a pandemic on March 11th, 2020 and a public health emergency of international concern (PHEIC) in late January 2020 by the World Health Organization (WHO). SARS-CoV-2 infects the respiratory tract causing flu-like symptoms and, in some, may cause severe illness like pneumonia and multi-organ failure leading to death. Today, Covid-19 cases almost reaching 9 million, with more than 450 thousand deaths. There is an urgent demand for developing a vaccine since no effective therapies or vaccines have been approved to this day to prevent or minimize the spread of the infection. In this review, we summarized the furthest vaccines in the clinical pipeline.

Keywords: Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), coronavirus disease 2019 (Covid-19), antibody dependent enhancement (ADE), receptor binding domain (RBD), pandemic, clinical trial, spike (S) protein

OPEN ACCESS

Edited by:
Denise L. Doolan, James Cook University, Australia

Reviewed by:
Rachel Graham, University of North Carolina at Chapel Hill, United States
Sofia A. Casares, Naval Medical Research Center, United States

***Correspondence:**
Abdullah M. Izmirly
amizmirli@kau.edu.sa
Elias K. Haddad
ee336@drexel.edu

Specialty section:
This article was submitted to Vaccines and Molecular Therapeutics, a section of the journal Frontiers in Immunology

Received: *14 May 2020*
Accepted: *13 July 2020*
Published: *19 August 2020*

Citation:
Alturki SO, Alturki SO, Connors J, Cusimano G, Kutzler MA, Izmirly AM and Haddad EK (2020) The 2020 Pandemic: Current SARS-CoV-2 Vaccine Development. Front. Immunol. 11:1880. doi: 10.3389/fimmu.2020.01880

CORONAVIRUS OVERVIEW

Coronaviruses are a group of enveloped viruses containing a positive-sense, single-stranded RNA genome (1). Coronaviruses originate from animals such as birds, bats, and camels (1–3) and may cause mild disease such as the common cold to severe illnesses in the respiratory track when infecting humans (4). In 2002, Severe Respiratory Acute Syndrome Coronavirus (SARS-CoV) and in 2012 Middle East Respiratory Syndrome were previous outbreaks that caused a great public health threat (5, 6). In December 2019, a novel strain of coronavirus was identified after an outbreak of pneumonia was reported in Wuhan, China (7). In February 2020, this virus was named "Severe Acute Respiratory Syndrome Coronavirus 2" (SARS-COV-2) by the Coronavirus Study Group (CSG) of the International Committee (8). SARS-CoV-2 causes a potentially fatal disease which the World Health organization has named "Covid-19" in February 2020 (9). Since June 23rd 2020, more than 8.9 million cases were reported worldwide with more than 469 thousand known deaths. Across the United states alone, nearly 2.3 million confirmed cases have been reported with more

than 119 thousand deaths making it the leading country in the number of Covid-19 confirmed cases and deaths followed by Brazil with more than 1 million cases and Russia with more than 599 thousand confirmed cases. Currently, the pandemic is underway and many more people are getting infected globally (10).

CLINICAL MANIFESTATIONS/SYMPTOMS

SARS-COV-2 infected individuals experience a wide range of symptoms which differs from one person to another. The clinical manifestations range from mild to severe illness and sometimes, death. Usually, symptoms develop anywhere from 2 days to 2 weeks after being exposed to the virus (11). The most common symptoms include fever, cough, shortness of breath, and fatigue (12, 13). Other symptoms include rhinorrhea, sputum production, headache, and sore throat. In addition, some individuals may display rare symptoms like gastrointestinal symptoms including diarrhea and vomiting. Many other symptoms may also develop such as hyposmia (reduced ability to smell) and hypogeusia (reduced ability to taste) (14). In more severe cases, individuals may require hospitalization and even admission into the intensive unit care (13, 15). Disease of these patients may quickly progress and cause complications such as acute respiratory distress syndrome (ARDS), sepsis shock, multiple organ failure, and secondary infection which may eventually lead to death in a short period of time (13, 15). Risk factors that contribute to more severe illness and critical conditions include age (the elderly over 65) and general health status (those with underlying disorders) including those with hypertension, cardiovascular disease, diabetes, and those with a weakened immune system. (13). Fortunately, many individuals experience only mild symptoms or were asymptomatic (16, 17). Symptoms are summarized in **Figure 1**.

TRANSMISSION

The beginning of the SARS-COV-2 pandemic has been traced back to the animal wholesale market in Wuhan, China where many Covid-19 cases first appeared after infected individuals visited that market (7, 18). It is believed that SARS-COV-2 likely originated from animals and that animal to human transmission is the main mechanism of transmission (7, 19). When the genome sequence of SARS-COV-2 was analyzed, it showed ∼90% similarity with bat SL-CoVZXC21 and bat-SL-CoVZC45 (20). This finding suggested that mammals are most likely to be the link between SARS-COV-2 and human. Other studies concluded that person-to-person may be the route of transmission since many Covid-19 cases were reported among family members and those that had been in contact with an infected person without any previous visits to the Wuhan animal market. It has shown that infected people could spread the virus even before any symptoms develop and asymptomatic individuals could spread the virus as well (21). There are many ways the virus can spread from person to person either through direct contact or through droplets when sneezing or coughing by an infected person (22). A study was done in different environmental conditions to evaluate the stability of SARS-CoV-2 in aerosols and on various surfaces. The study data showed that SARS-CoV-2 remained viable for 3 h in aerosols. On cardboard, the virus remained viable for 24 and 72 h on stainless steel and plastic. In addition, the virus viability lasted 4 h on copper (23). The severity level of the SARS-CoV-2 disease is much less compared to MERS and SARS-CoV, however, the infectiousness level of SARS-CoV-2 is much higher than other coronaviruses, which probably is due to viral shedding, incubation time, and binding strength to its receptor, angiotensin-converting enzyme 2 (ACE2). The infectiousness of SARS-CoV-2 resembles influenza more than SARS-CoV since the infectiousness reaches its highest level shortly around or even before the onset of symptom. In other words, the infected patients spread SARS-COV-2 before they have developed symptoms due to the rapid shedding of the virus which begins 2–3 days before the appearance of the first symptoms. Then, the viral load decreases significantly after 8 days of developing the onset of symptoms (24). Recently, a group identified a unique furin cleavage site at the S1/S2 boundary of SARS-CoV-2 S protein setting it apart from SARS-CoV (25). It is possible that this furin cleavage motif has contributed to the expanded tropism and transmission of SARS-CoV-2 and the high affinity binding to ACE2. It should be noted, that since individuals who are asymptomatic spread the virus, and the virus spread could cause pandemic within weeks, thus prevention precautions such as quarantine and isolation are difficult to achieve. This evidence leads to the belief that a vaccine is an extremely important goal

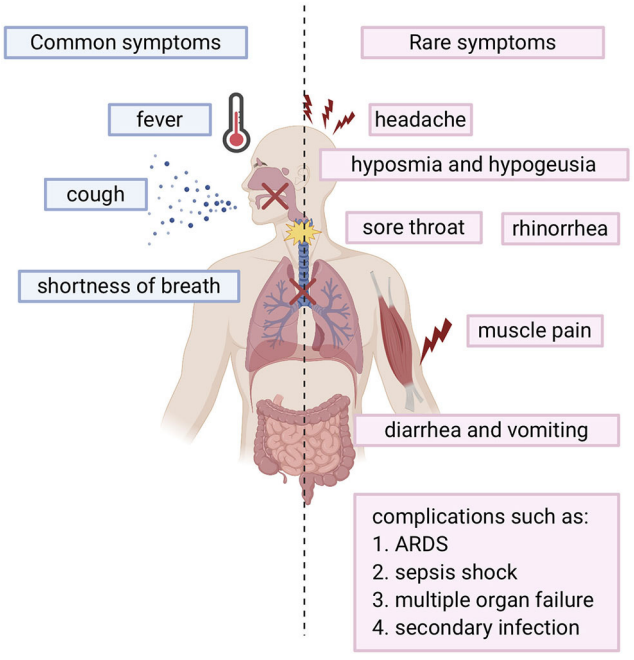


FIGURE 1 | Overview of COVID-19 symptoms: Blue bars represent the common symptoms. Pink bars represent the rare symptoms.

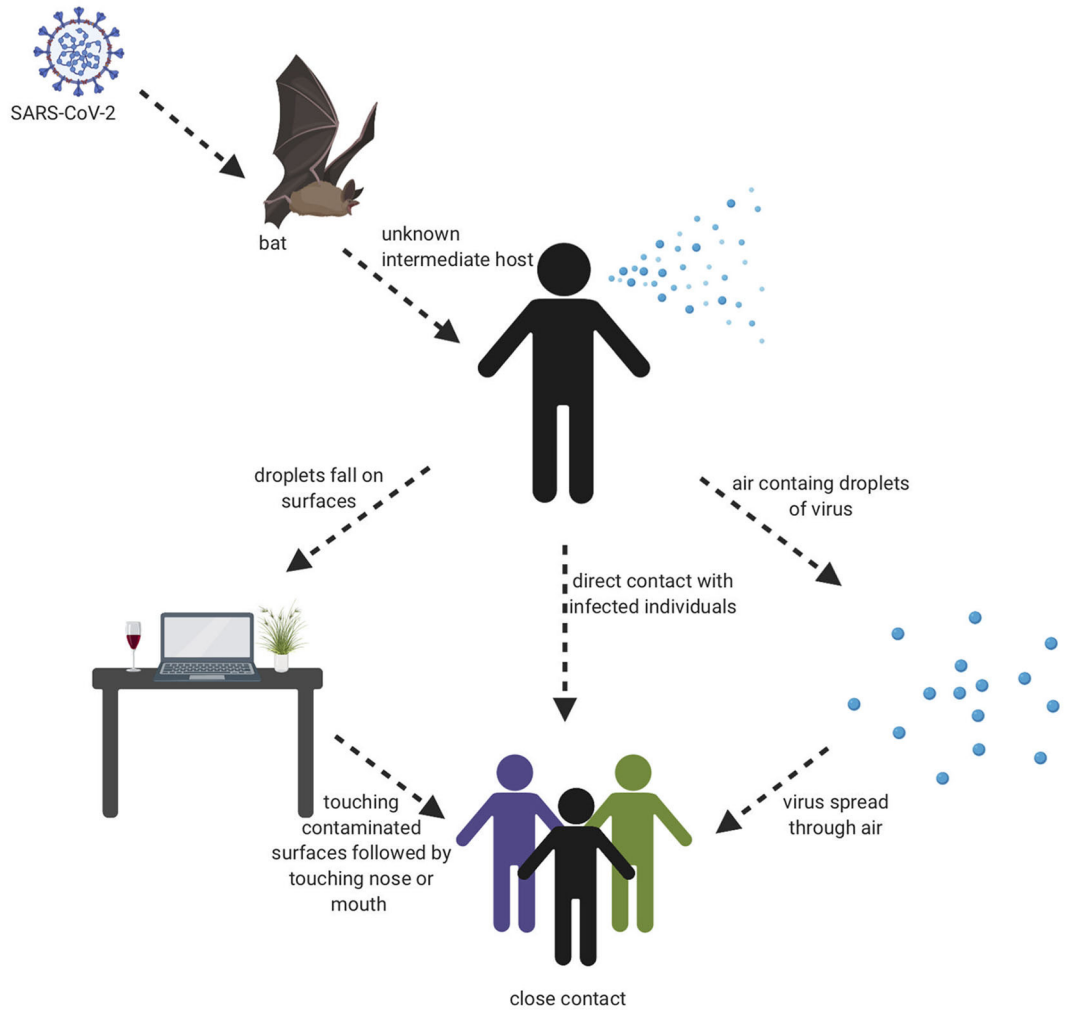


FIGURE 2 | Transmission cycle of SARS-CoV-2. It is believed that animal to human transmission is the main cause of infection through consuming an infected animal as a source of food. Then, the infection can spread through person to person, aerosols, and touching contaminated surfaces.

to prevent future spread of this disease. Visual depiction of SARS-COV-2 transmission is shown in **Figure 2**.

STRUCTURE AND LIFE CYCLE

SARS-CoV-2 is a single stranded RNA virus that belongs to the Betacoronavirus (betaCoVs) genus. It has a diameter of ∼60–140 nm in size and a genome of 29,891 nucleotides that encodes for 9,860 amino acids. Studies have shown that SARS-CoV-2 shares ∼80% of its sequence identity with SARS-CoV (26). This genomic analysis suggests that this virus may have originated and evolved from bats. The virus's genome encodes for ∼16 non-structural proteins (NSP) and 4 major structural proteins. The structural proteins include the spike (S) protein, the envelope (E) protein, membrane (M) protein, and the nucleocapsid (N) protein (26). The S protein mediates the binding of SARS-CoV-2 to ACE2 on the host cell which leads to virus entry and pathogenesis. The S protein consists of two subunits (S1 and S2). S1 subunit carries the receptor binding domain (RBD) that directly interacts with the ACE2 receptor, while S2 subunit mediates the membrane fusion of the virus-host cell by containing the essential elements required for this process (26). The N protein forms the nucleocapsid and is responsible for mRNA transcription and RNA replication. It is also responsible for the virus budding through signal transduction (27). The M protein plays a role in the assembly of the virus, while the E protein is considered to be a major virulence factor and plays a role in the secretion of inflammatory factors (28, 29).

SARS-CoV-2, like its antecedent SARS-CoV, spreads through respiratory droplets and through fomite transmission (23). After exposure, the viral S proteins bind to ACE2 receptors that are broadly expressed in a variety of cells including respiratory epithelia and alveolar monocytes and macrophages as well as myocardial, renal, hepatic, and gastrointestinal tract tissues (30–32). SARS-CoV-2 may also utilize CD209L and CD147 as an

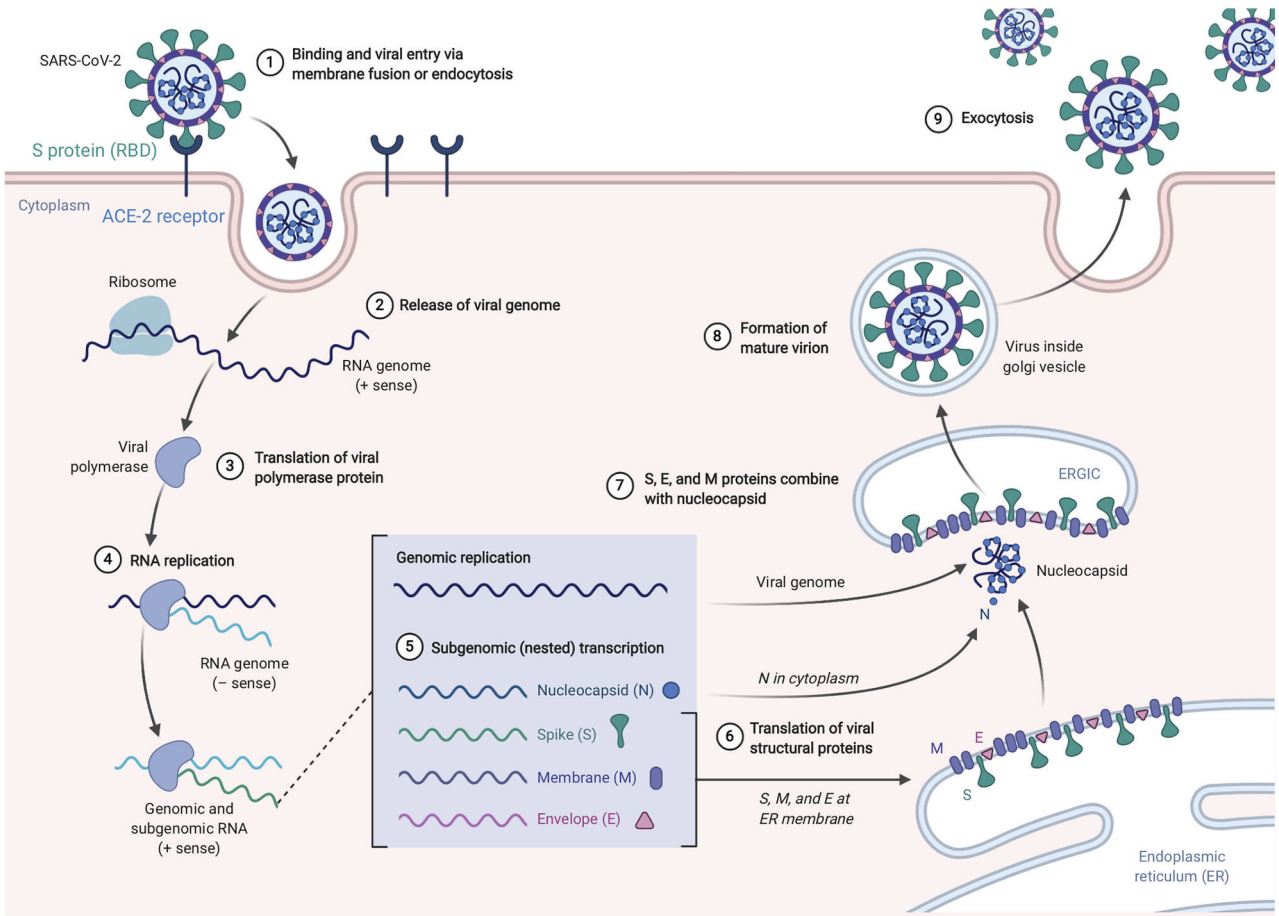


FIGURE 3 | SARS-Cov-2 life cycle. The virus enters and replicates in cells that express ACE2 such as lung epithelia, myocardial, renal, GI tract, and hepatic cells. The mature virions are released from the primary host cells via exocytosis and infect new target cells. Figure from Biorender.com.

alternative receptors like SARS-CoV though with much lower affinity (33, 34). The use of these alternate receptors may partially explain why the transmission rate of SARS-CoV-2 is so high as they will allow for potent infectivity even on cells expressing low ACE2. This entry mechanism depends upon cellular proteases including human airway trypsin-like protease (HAT) (35), transmembrane protease serine 2 (TMPRSS2) (36), and cathepsins. These proteases function to split the spike protein for further penetration. Following fusion of the viral envelope to the host membrane, the viral RNA is released into the cytoplasm. Open reading frame 1a (ORF1a) and ORF1b are then translated into the overlapping polyproteins, pp1a and pp1ab which are cleaved by the viral papain-like proteases and a serine type Mpro (chymotrypsin-like proteases) that are encoded by ORF1a to produce 16 non-structural proteins that form the RNA replicase-transcriptase complex (RTC). Viral RNA synthesis produces both genomic and sub-genomic RNAs, the latter which serves are mRNA for 7–9 structural proteins such as the E, N, M, and S proteins that are produced through discontinuous transcription (37). Both genomic and sub-genomic RNAs are produced through negative-sense (-RNA) intermediates via the RNA-dependent RNA polymerase (RdRp) (1). The viral nucleocapsids are then assembled with N-protein encapsidated genomic RNA in the cytoplasm. The assembled viral nucleocapsid buds into the lumen of the endoplasmic reticulum-Golgi intermediate compartment (ERGIC) and the completed, mature virion is released from the infected cell through exocytosis (38). Depiction of the life cycle is shown in **Figure 3**.

Because of the urgent need for a vaccine, WHO accelerated the vaccine development process by activating the R&D Blueprint that aims to diminish the time of research and development process, improve scientists and global health professionals coordination, and generate new norms and standards to learn from the global response and its improvement (39). Currently, more than 115 vaccine candidates are being developed. The majority are in exploratory and preclinical stages however, around ten vaccine candidates have advanced recently and moved into clinical development (**Table 1**). These include mRNA-1273, developed by Moderna, Ad5-nCoV, developed by CanSino Biologicals, INO-4800, developed by Inovio, LV-SMENP-DC, and pathogen-specific aAPC, developed by

TABLE 1 | List of clinical-phase vaccine candidates for COVID-19 and clinical trial status as of June 2020.

Phase I				
Name	**Developer**	**Method**	**Trial**	**Trial enrollment**
bacTRL-spike	Symvivo corporation	Orally administered probiotic bacteria encoding the SARS-COV-2 S protein	NCT04334980	Not yet recruiting
NVX-CoV2373	Novavax	Recombinant S protein made using Novavax's proprietary nanoparticle technology and includes Matrix-M adjuvant	NCT04368988	Recruiting
INO-4800	Inovio pharmaceuticals	DNA vaccine administered intradermally followed by CELLECTRA® electroporation (EP)	NCT04336410	
AZD1222 nCoV-19 formerly known as (ChAdOx1)	University of oxford	Chimpanzee adenovirus vector carrying gene for the SARS-COV-2 S protein	NCT04324606	
mRNA-1273	Moderna	Lipid nanoparticle encapsulating mRNA for SARS-COV-2 S protein	NCT04283461	
Covid-19 aAPC	Shenzhen geno-immune medical institute	Lentiviral vector expressing SARS-COV-2 proteins and immunomodulatory genes to modify artificial antigen presenting cells and active T cells	NCT04299724	
LV-SMENP DC			NCT04276896	
BNT162	Pfizer and BioNTech	Lipid nanoparticle encapsulating mRNA for SARS-COV-2 S protein	NCT04368728	
Phase II				
Ad5-nCoV	CanSino Biologicals	Adenovirus encoding the SARS-COV-2 S protein	NCT04341389	Active, not recruiting
Phase III/IV				
BCG vaccine	Ain shams university	Intradermal or intracutaneous administration of BCG vaccine to induce non-specific protective effect on SARS-COV-2	NCT04350931	Not yet recruiting
	UMC utrecht		NCT04328441	Recruiting
	murdoch children's research institute		NCT04327206	
	Texas A&M university		NCT04348370	
	Bandim health project		NCT04373291	Not yet recruiting
	Hellenic institute for the study of sepsis		NCT04414267	Recruiting
	Assistance publique–hôpitaux de paris		NCT04384549	Not yet recruiting
	Universidad de antioquia		NCT04362124	
	Radboud university		NCT04417335	Active, not recruiting
	University of Campinas, Brazil		NCT04369794	Not yet recruiting
	TASK applied science		NCT04379336	Recruiting

Shenzhen Geno-Immune Medical Institute, and AZD1222 nCoV-19 formerly known as ChAdOx1, developed by the University of Oxford (**Figure 4A**). Numerous other companies and vaccine developers are on their approach to initiate human testing in a couple of months including Viroclinics Xplore. Various vaccine platforms are being used, including live attenuated virus, inactivated virus, peptide-based, virus-like particles, replicating/non-replicating viral vector, recombinant protein, and nucleic acid (DNA and RNA) (**Figure 4B**) (40–46). Following the public release of the complete sequence of the SARS-CoV-2 genome on January 12, 2020, numerous nucleic acid-based vaccine candidates have emerged including

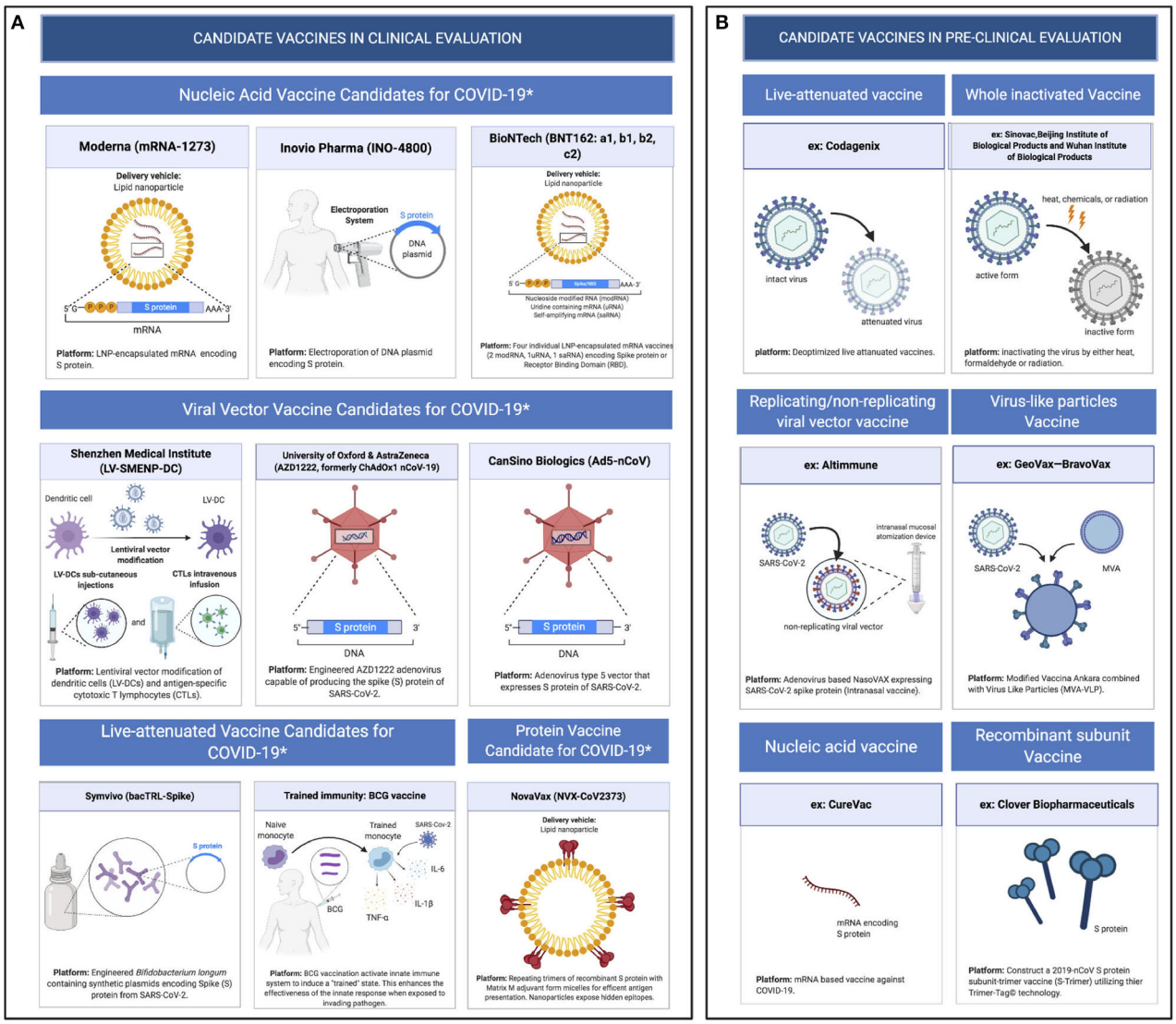


FIGURE 4 | Pipeline of COVID-19 vaccine candidates' platforms. **(A)** Ongoing vaccine development platforms for SARS-CoV-2 (live attenuated, nucleic acid, viral vector, and protein vaccines). **(B)** Pre-clinical vaccine candidates' platforms (live attenuated virus, inactivated virus, replicating/non-replicating viral vector, virus like particles, subunit, and nucleic acid vaccine).

mRNA-1273, INO-4800, and AZD1222 nCoV-19 (47). Most of nucleic acid vaccines are based on the major antigen S protein coding sequence. RBD on S1 can recognize different type of receptors (48). For instance, MERS-CoV recognizes dipeptidyl peptidase 4 (DPP4) as its receptor whereas SARS-CoV recognizes ACE2 (49). It has been reported that the sequence and the structure of SARS-CoV-2 is more like SARS-CoV than MERS-CoV however, SARS-CoV-2 S protein shares a global protein fold architecture with the MERS-CoV S protein (50). Despite the strong structural homology between the two RBDs of SARS-CoV and SARS-CoV-2, studies demonstrated a limited antibody cross reactivity between SARS-CoV RBD-specific monoclonal antibodies to 2019-nCoV S protein (51, 52). Therefore, developing a vaccine targeting RBD can inhibit viral attachment hence, fusion.

CURRENT VACCINES

Phase I Clinical Trials

Symvivo

Symvivo Corporation is leading an ongoing Phase I clinical trial evaluating the safety, tolerability, and immunogenicity of their bacTRL-spike vaccine for Covid-19 prevention (NCT04334980). Once orally administered, the bacTRL-spike vaccine is designed such that the genetically modified probiotic bacteria, *Bifidobacterium longum,* should colonize the gut,

bind to intestinal epithelial cells, replicate, secrete, and deliver plasmids expressing the SARS-CoV-2 spike protein. As a living-medicine, the expression of the SARS-CoV-2 S protein is expected to be sustained throughout the life of the colonized *B. longum.* This results in continued delivery and expression of SARS-CoV-2 S protein encoding plasmids. Translation of this plasmid within the gastrointestinal lymphoid tissues is expected to initiate a robust mucosal, systemic humoral, and cell-mediated immune response in treated patients. This Phase I clinical trial will enroll 84 healthy adult volunteers who will receive 1, 3, or 10 billion colony forming units (CFU) of the live, genetically modified *B. longum* alongside placebo treated control patients. Unlike typical vaccine administration, the bacTRL-spike vaccine will be administered as an oral, lyophilized gel-capsule similar to traditional consumer probiotic supplements (53). Patients will be monitored over the course of 12 months to measure outcomes including but not limited to the production, seroconversion, and stability of SARS-CoV-2 S antibodies, intestinal colonization of the genetically modified *B. longum*, and frequency of adverse events. This study is being conducted at BC Children's Hospital Research Institute and The Canadian Center for Vaccinology and is anticipated to be completed by December 31, 2021.

Novavax

NVX-CoV2373, a vaccine being developed by Novavax, is a recombinant S protein (SARS-CoV-rS) vaccine that is made using Novavax's proprietary nanoparticle technology and includes Novavax's Matrix-M saponin-based adjuvant. Matrix-M is a potent inducer of leukocyte migration into the draining lymph nodes (LN) resulting in the increase in T-, B-, NK, and dendritic cells in draining LNs. These recruited cells also showed an upregulation of activation markers (54, 55). This adjuvant, when compared with other adjuvants like Alum, AS03, and FCA, is a more potent alternative (56). In pre-clinical trials, NVX-CoV2373 was shown to be highly immunogenic in animal models. NVX-CoV2373 produced high levels of S protein-specific antibodies that can block ACE-2 human RBD and wild-type SARS-CoV-2 neutralizing antibodies after one dose. After a second dose, the neutralization titers jumped eight-fold (57). NVX-CoV2373 will enter clinical trials in mid-May and will undergo a Phase I/Phase II approach (NCT04368988). This study will first enroll 131 subjects to assess the dosage amounts and the number of doses required with and without Matrix-M adjuvant. The study will start with 5 or 25 μg of SARS-CoV-rS with or without equal amounts of Matrix-M. It will also look at 25 μg SARS-CoV-rS with 5μg of Matrix-M. Each arm of this study will receive two intramuscular (IM) injections at a day 0 and day 21. Primary outcome measures will include looking at frequency of adverse events and serum IgG antibody levels specific for SARS-CoV-2 rS protein.

Inovio

INO-4800 is a synthetic DNA-based vaccine developed by Inovio pharmaceuticals collaborating with researchers at the Wistar Institute, Seoul National University Hospital, and the International Vaccine Institute (IVI). INO-4800 vaccine is a synthetic DNA-based vaccine that are delivered into human cells via electroporation (EP) and translated into S proteins to induce an immune response. The main advantage of the nucleic acid-based platform is an accelerated developmental timeline due to their ability to be designed rapidly, manufactured in large quantities, and have great flexibility in terms of antigen manipulation (58, 59). In pre-clinical trials, immunizing animal models with INO-4800 has shown to rapidly provoke the stimulation of T cells and potent RBD binding antibodies following a single immunization (60). Muthumani et al. have extensive experience with DNA vaccine against coronavirus as they have demonstrated the ability of a DNA vaccine encoding the MERS S protein (INO-4700) to induce a potent cellular immunity, antigen-specific neutralizing antibodies, and provide protection in NHP challenge models (61). INO-4800 utilizes a strategy identical to the DNA vaccine for MERS INO-4700. Currently, INO-4700 is in clinical testing in week 16 from a Phase 1/2a trial in South Korea. INO-4700 clinical tests outcome showed very promising results in which 92% of the vaccine recipients displayed the ability to neutralize the virus and 84% of vaccine recipients, after the third dose of 0.6 mg of INO-4700, showed robust T cell responses. From the previous studies, INO-4800 shows a promising vaccine candidate against SARS-CoV-2. DNA vaccines are safe since they are unable to revert into active forms. Besides offering long-term stability for ease of storage and transport (cold chain free), DNA vaccines induce strong cellular and humoral responses making the DNA vaccine an ideal approach. Inovio started testing *in-vivo* and *in-vitro* expression and immunogenicity of INO-4800 just 6 weeks after SARS-CoV-2 genome sequence identification. Currently, it has entered a phase 1 clinical trial (NCT04336410) to evaluate its safety, tolerability, and immunological profile. It will be administered intradermally (ID) on day 0 and week 4 of 1.0 mg per dosing visit followed by electroporation (EP) using the CELLECTRA® 2,000 device in healthy adult volunteers (60).

University of Oxford

AZD1222, formally ChAdOx1, nCoV-19 vaccine, developed in the UK by Jenner Institute of University of Oxford, contains the genetic sequence of the SARS-COV-2 S protein with a transgenic, non-replicating chimpanzee adenovirus-based vector (62). This viral vectored vaccine platform has a great advantage since it leads the host cells to express the coronavirus S protein thus leading to the stimulation and production of a robust humoral and T cell-mediated immune response upon immunization (63). This platform is currently being used for a MERS vaccine and has completed phase I clinical trial (NCT03399578) (64, 65). The SARS-COV-2 vaccine trial is now recruiting for phase I/II combined clinical trial (NCT04324606) (62, 66). The non-replicating feature of this vaccine makes it relatively safe in individuals with underlying diseases and children (67). In this trial, a total of 1112 healthy volunteers aged 18–55 years will be enrolled. In this study, viral particles (vp) of AZD1222 nCoV-19 vaccine will be delivered to the experimental groups with the Meningococcal conjugate vaccine (MenACWY) used as a control. Volunteers will be divided into 4 groups (Groups: 1a, 1b, 2a, 2b, 3, 4a, 4b, 4c, 4d). Experimental groups 1a, 2a, 4a will receive a single dose of 5×10^{10}vp AZD1222 nCoV-19

while group 3 will receive one dose of 5×10^{10}vp AZD1222 nCoV-19 at week 0 and one dose of 2.5×10^{10}vp AZD1222 nCoV-19 at week 4. Group 4c will receive a single dose of 5×10^{10}vp AZD1222 nCoV-19 plus Paracetamol. In addition, active comparator groups 1b, 2b, 4b, and 4d will receive a standard single dose of MenACWY (IM) plus Paracetamol for group 4d. Paracetamol is an antipyretic/analgesic drug known to reduce fever and pain (febrile reactions) and is generally used after vaccination. However, in this trial the main purpose of using this drug was to assess safety, reactogenicity, immunogenicity, and efficacy for participants receiving prophylactic Paracetamol. It is important to include this arm of the study because it has been shown that prophylactic Paracetamol administration at the time of vaccination or during the first 6–8 h post vaccination could impact the immune response negatively to several vaccine antigens in children and adults (66, 68–70). The time frame of this study will be ∼6 months with a follow up visit at Day 364 (66). Primary outcome measures will be to assess the efficacy and adverse events of AZD1222 nCoV-19. Secondary outcome measures will be to assess the safety, tolerability, and reactogenicity profiles of the candidates (66).

Moderna

mRNA-1273 is created by Moderna Inc in collaboration with National Institute of Allergy and Infectious Diseases (NIAID) and Coalition for Epidemic Preparedness Innovations (CEPI). It is similar to INO-4800 where both vaccines encode the S protein of SARS-CoV-2 that is translated by host cells following vaccination and will mimic a natural infection immune response. In this study, the developers used a novel mRNA encapsulated within lipid nanoparticles (LNPs) composed of ionizable lipid, distearoyl phosphatidylcholine, cholesterol, and polyethylene glycol lipid. Vaccination by mRNA/LNP is a novel approach that has improved its ability to elicit strong immune responses in numerous pre-clinical and clinical studies developed by Moderna including CMV, Zika virus, H7N9, hMPV, and RSV (71, 72). Formulation of the mRNA antigen within an LNP improves immunogenicity, protecting the mRNA from enzymatic degradation, and facilitating efficient uptake by target cells. Moderna started its clinical trial just 42 days after the complete viral sequence has been available (NCT04283461). The study is recruiting 155 healthy males and non-pregnant females, starting at 18–99 years of age. In the primary objective, individuals will be enrolled into one of thirteen cohorts (10, 25, 50, 100, 250 μg) and will receive the vaccine IM on days 1 and 29 of 0.5 milliliter of mRNA-1273 to assess the vaccine safety and reactogenicity. If the vaccine passes the primary objective and meets all safety and reactogenicity criteria, it will undergo the secondary objective to evaluate the immunogenicity. The outcome measures for this trial centers around the frequency of adverse events and determines the antibody response and percentage of subjects who have seroconverted. In May, Moderna announced positive interim phase one data for mRNA-1273. All participants among the groups who received 25 and 100 μg dose cohorts seroconverted with binding antibody levels which reached or exceeded what is seen in convalescent sera following the second dose of vaccination. Eight of these seroconverted volunteers had neutralizing antibody titers at or beyond what is generally seen in convalescent sera. In addition, viral challenge studies in mice showed complete protection against viral replication in their lungs after vaccination with mRNA-1273. This immunogenicity increased in a dose-dependent manner that was seen in all 45 participants at the three dose levels, and it was primed and boosted within the 25 and 100 μg dose levels. The results showed that mRNA-1273 is generally safe and well-tolerated. However, a few of the participants experienced grade 3 adverse events such as erythema around the injection site which were transient and self-resolving. No grade 4 or serious adverse events have been noticed. The age of the participants should be considered for the positive results due to the fact that usually younger individuals tend to show better response to the vaccine than if they were mostly from the senior group who are at a higher risk. The potential advantages of an mRNA approach include the ability to mimic natural infection, stimulate a potent immune response by combining multiple mRNAs into a single vaccine, and rapid discovery and design for a quicker respond to emerging pandemic threats (73, 74). Recently, on May 7th 2020, Moderna received an approval from the FDA to begin phase two studies which are expected to begin soon.

Shenzhen Geno-Immune Medical Institute

Antigen-specific T cells have a crucial role in a variety of diseases including viral infection. Generating large quantities of viral specific T cells in a rapid manner may eradicate the invasion of SARS-CoV-2. One effective method to generate massive amounts of T cells is through developing a genetically modified, artificial antigen presenting cell (aAPC) expressing SARS-COV-2-specific antigens. This is accomplished by using lentivirus vectors (LV) expressing SARS-COV-2 minigene (SMENP) and immune modulatory genes. LV are used to provoke the naïve T cells leading to effector T cell differentiation and proliferation. Dendritic cells are the ideal LV vaccine targets as they are the most potent antigen presentation cells (APCs) in which they are able to stimulate robust and durable antigen-specific T cell responses (75). LV-SMENP-DC and pathogen-specific Artificial Antigen-Presenting Cells (aAPC) are two vaccines developed by Shenzhen Geno-Immune Medical Institute, China which are based on APCs ability to stimulate viral antigen-specific T cells. Both vaccines are currently in Phase l clinical trial to evaluate their safety and reactogenicity. One hundred healthy volunteers and SARS-COV-2 infected patients will be receiving 5×10^6 cells of LV-DC alone via ID injection or 5×10^6 cells of LV-DC vaccine and 1×10^8 antigen-specific cytotoxic T lymphocytes (CTLs) via ID injection and IV infusion, respectively (NCT04299724, NCT04276896) (76, 77). The primary and secondary outcome measures for these trials focus on the frequency of adverse events, mortality rates, and subjects with positive T cell responses.

Pfizer and BioNTech

BioNTech and Pfizer have started their first clinical trial for SARS-COV-2 vaccine candidate, BNT162, in Germany on April 23rd 2020 (NCT04368728). Phase I/II will enroll 200 healthy

participants aged 18–55 and will receive a dose range of 1 to 100 μg. On May 5th 2020, Phase I/II clinical trial for the BNT162 vaccine program has been started in the U.S. In Phase I/II trial, 360 healthy subjects in U.S. will enroll and will be divided into two age cohorts (18–55 and 65–85 years of age). In addition, the company has partnered with Fosun Pharma to begin clinical trials of SARS-COV-2 vaccine BNT162 in China. Like mRNA-1273 from Moderna, BNT162 is a lipid nanoparticle encapsulating mRNA encoding for SARS-CoV-2 antigens. The company's development program involves four vaccine candidates, each of them demonstrating a different combination of mRNA format and target antigen (78). The primary outcome measures for this study include reports of adverse events while the secondary outcome measures focus neutralizing antibody levels.

Phase II Clinical Trial

CanSino Biologics

In conjunction with CanSino Biologics, the Beijing Institute of Biotechnology (BiB) and the Jiangsu and Hubei province Centers for Disease Control and Prevention have developed a recombinant adenovirus Type 5 Vector, Ad5-nCoV, SARS-CoV-2 vaccine candidate. Ad5-nCoV contains a replication-defective adenovirus type 5 as a vector to express the full-length SARS-CoV-2 S protein (79). After successfully moving through Phase I clinical trial, Ad5-nCoV has become the first SARS-CoV-2 vaccine to move into Phase II (NCT04341389). As of review publication, CanSino Biologics have found that this vaccine in phase I trial was tolerable and immunogenic at 28 days post-vaccination with humoral responses against SARS-CoV-2 peaking at day 28 in healthy adults. Cellular responses were noted from day 14 post-vaccination though it is not yet known whether the T or B cell responses are protective (80). During phase 2,500 subjects will be enrolled, 250 subjects in the middle-dose vaccine group (5 × 1011 vp) and 125 subjects in the low-dose (1 × 1011 vp) and placebo groups, respectively. Immunogenicity will be tested on days 0, 14, 28, and 6 months post-vaccination. The vaccine will be administered through IM injections at day 0. The primary outcome measure is the occurrence of adverse events, antibody responses, specifically neutralizing antibody levels 0–14 days post-vaccination. Secondary outcome measures also include the occurrence of adverse events and antibody levels with a longer time frame (0–28 days post-vaccination). Using Ad5 as a vector comes with a potential issue known as the Ad5 set-back. Because adenoviruses cause the common cold and the average person has statistically been infected before, the presence of pre-existing immunity from natural exposure to Ad5 can dampen cellular immune responses to whatever antigens are encoded by the vector vaccine. Other Ad5 vaccines have tried to overcome this set-back using potent prime-boost regimens though the Ad5-nCoV does not appear to take advantage of this technique to overcome this potential pre-existing Ad5 immunity (81). CanSino Biologics does note in their phase one results that there is a negative effect on the pattern of T cell responses due to Ad5 pre-exiting immunity.

Phase III Clinical Trial

BCG Vaccines

There are several ongoing Phase III (NCT04327206) or IV (NCT04348370) clinical trials evaluating the ability of Bacillus Calmette-Guérin (BCG) vaccines to prophylactically protect health care workers, healthy adults, and elderly populations against Covid-19. BCG vaccines are being considered to reduce the impact of Covid-19 due to its ability to induce trained immunity. Trained immunity involves the induction of metabolic and epigenetic modifications that promote an innate immune response against subsequent infections. Through trained immunity, BCG vaccines have been shown to prevent respiratory infections such as pneumonia and influenza in children and the elderly (82, 83). BCG vaccines have also been shown to reduce the severity of other viral infections. This was demonstrated in a human yellow fever infection model where BCG vaccination reduced viremia and in mouse studies where BCG vaccination reduced the severity of mengovirus infection (84–86). This non-specific protection induced by BCG vaccines may act as a stopgap while SARS-COV-2 specific vaccines are being developed (82). Patients included in these trials will receive BCG vaccines containing either the TICE, Danish 1331, or Moscow 361-1 strains of live attenuated *Mycobacterium bovis*. Patients enrolled will receive either 0.1 mL of the BCG vaccine, 0.1 mL of a saline solution as a placebo, or no immunization. BCG vaccine dosages will include 2 × 10^5 − 8× 10^5 CFUs, 2 × 10^5 CFUs, or 1 × 10^5−33 × 10^5 CFUs of *M. bovis* depending on the strain of being used. Vaccine administration will either be intradermal or intracutaneous depending on the trial. The primary outcome across most trials is Covid-19 incidence as measured by detecting SARS-CoV-2 by PCR or detection of SARS-CoV-2 Spike protein antibodies and hospital admittance. Several secondary outcomes being evaluated include but are not limited to Covid-19 symptom duration, disease severity and deaths. Study enrollment estimates vary with the lowest including 500 patients and highest including 10,078 patients. In addition to enrollment variation, study completion dates vary with the soonest estimated completion date of December 1, 2020 and latest of May 2022.

ANTIBODY DEPENDENT ENHANCEMENT (ADE): A POTENTIAL HURDLE FOR CORONAVIRUS VACCINE DEVELOPMENT

Due to the presence of different strains of coronavirus and the strong structural homology between the two RBD of SARS-CoV and SARS-CoV-2, the cross-reactivity between antibodies of different coronaviruses must be taken into consideration for SARS-CoV-2 vaccine development. Antibodies have a dual role in controlling infections in which either they neutralize the infection or enhance pathogen uptake (87). Several viruses rely on pre-existing antiviral antibodies for their entry into the target cells, a mechanism known as antibody-dependent enhancement (ADE). Pre-existing antiviral antibodies from heterologous strains can prevent the virus entry to the cells by

blocking the binding to its natural receptor on the host cell surface. However, these antibodies could facilitate the entry of the virus to host cells through either interaction of the antibody-virus complex with FcR receptors on various immune cells or complement receptors by activating the complement classical pathway (88). Both mechanisms tend to be linked to disease exacerbation. Generally, ADE educes sustained inflammation, lymphopenia, and potentially, cytokine storm, causing severe illness, or death. Furthermore, ADE has been observed in a variety of viruses including flaviviruses, HIV, and Ebola virus (89, 90). Importantly, ADE has been extensively studied in dengue viral infections since ADE has been linked to the severity of dengue shock syndrome (91). It should be noted that ADE was linked to some vaccines, as this was demonstrated in the efficacy trials of the tetravalent dengue vaccine (CYD-TDV). In the CYD-TDV trial, they found that seronegative individuals who received CYD-TDV suffered severe dengue disease that mimics the natural secondary infection unlike seropositive individuals who had been exposed to dengue before vaccination (92).

Recently, a study demonstrated that ADE occurs not only through the typical mechanism of the presence of sub-neutralizing antibodies but also that neutralizing antibodies against RBD might be involved in ADE. This mechanism depends on the affinity, the amount, and the specificity of the antibodies (93). Furthermore, from a different group, the cross reactivity of anti-RBD polyclonal antibodies specific for SARS-CoV with RBD protein of SARS-CoV-2 pseudovirus was demonstrated (50). Moreover, ADE phenomena have been identified in SARS-CoV infections, and now potentially COVID-19. It could be hypothesized that ADE has a role in the high mortality rate in China (94).

The high mortality rate in some countries over the others could be due to prior exposure of one or more mild strains of similar coronaviruses. The data obtained from patients of Hubei region showed lymphopenia and sustained inflammation in most of the severe and death cases (95). Based on the previous information, individuals suffering the most severe disease of COVID-19 may experience the effects of antibody dependent enhancement (ADE). ADE as a complication of COVID19 should be at the forefront while developing SARS-COV-2 vaccines to avoid similar mistakes in other vaccine development like the dengue vaccine (87, 92).

CONCLUDING REMARKS

In this review, we summarized the background and pathogenesis of SARS-CoV-2 and the front-runners in the race of SARS-COV-2 vaccine development. Currently, we are living in unprecedented times with a rapidly evolving situation and uncovering new insights about the virus every day. Due to this situation, an accelerated track for vaccine development has been put in motion. However, it is important to not jeopardize the safety of the vaccines produced and to not undermine the ADE risk factor that is known to be associated with coronaviruses (96, 97). There are many unanswered questions that need to be addressed regarding SARS-COV-2 including how the presence of antibodies will impact the clinical course and severity of the disease. We also need to determine if infection will protect you from future infection and if so, how long protection will last and what the correlates of that protection entail. We suggest harnessing the ability of a certain T helper cell subset called T follicular helper (Tfh) cells that are essential for high affinity antibodies and class switching (87). Taking what is known about ADE and coronaviruses, researchers should proceed with extreme caution and keep ADE into consideration while we move forward in SARS-COV-2 vaccine development.

AUTHOR CONTRIBUTIONS

SawA, JC, and GC contributed to writing sections of the paper and figure design. EH, AI, MK, and SanA contributed to writing up the paper. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by NIH funding as part of Human Immune Project Consortium (HIPC) to EH # U19 AI128910 and AI125202. This work was also supported by the Drexel University COVID-19 Rapid Response Fund to MK and EH.

ACKNOWLEDGMENTS

The authors would like to acknowledge King Abdulaziz University and thier fellowship program for their support. Figures were created using Biorender.com.

REFERENCES

1. Fehr AR, Perlman S. Coronaviruses: an overview of their replication and pathogenesis. *Methods Mol Biol.* (2015) 1282:1–23. doi: 10.1007/978-1-4939-2438-7_1
2. Azhar EI, El-Kafrawy SA, Farraj SA, Hassan AM, Al-Saeed MS, Hashem AM, et al. Evidence for camel-to-human transmission of MERS coronavirus. *N Engl J Med.* (2014) 370:2499–505. doi: 10.1056/NEJMoa1401505
3. Ren W, Li W, Yu M, Hao P, Zhang Y, Zhou P, et al. Full-length genome sequences of two SARS-like coronaviruses in horseshoe bats and genetic variation analysis. *J Gen Virol.* (2006) 87:3355–9. doi: 10.1099/vir.0.82220-0
4. Wevers BA, van der Hoek L. Recently discovered human coronaviruses. *Clin Lab Med.* (2009) 29:715–24. doi: 10.1016/j.cll.2009.07.007
5. Peiris J, Guan Y, Yuen K. Severe acute respiratory syndrome. *Nat Med.* (2004) 10:S88–97. doi: 10.1038/nm1143
6. Zaki AM, Van Boheemen S, Bestebroer TM, Osterhaus AD, Fouchier RA. Isolation of a novel coronavirus from a man with pneumonia in Saudi Arabia. *N Engl J Med.* (2012) 367:1814–20. doi: 10.1056/NEJMoa1211721
7. Zhu N, Zhang D, Wang W, Li X, Yang B, Song J, et al. A novel coronavirus from patients with pneumonia in China, 2019. *N Engl J Med.* (2020) 382:727–33. doi: 10.1056/NEJMoa2001017
8. Coronaviridae Study Group of the International Committee on Taxonomy of Viruses. The species severe acute respiratory syndrome-related coronavirus:

classifying 2019-nCoV and naming it SARS-CoV-2. *Nat Microbiol.* (2020) 5:536–44. doi: 10.1038/s41564-020-0695-z

9. World Health Organization. *WHO Director-General's Remarks at the Media Briefing on 2019-nCoV on 11 February 2020*. (2020). Available online at: https://www.who.int/dg/speeches/detail/who-director-general-s-remarks-at-the-media-briefing-on-2019-ncov-on-11-february-2020 (accessed June 22, 2020).
10. World Health Organization. *Coronavirus Disease(COVID-19) Situation Report – 155.* (2020). Available online at: https://www.who.int/docs/default-source/coronaviruse/situation-reports/20200623-covid-19-sitrep-155.pdf?sfvrsn=ca01ebe_2 (accessed June 23, 2020).
11. Centers for Disease Control and Prevention (CDC). *Coronavirus Disease 2019 (COVID-19).* (2020). Available online at: https://www.cdc.gov/coronavirus/2019-ncov/symptoms-testing/symptoms.html (accessed June 23, 2020).
12. Ren LL, Wang YM, Wu ZQ, Xiang ZC, Guo L, Xu T, et al. Identification of a novel coronavirus causing severe pneumonia in human: a descriptive study. *Chinese Med J.* (2020) 133:1015–24. doi: 10.1097/CM9.0000000000000722
13. Wang D, Hu B, Hu C, Zhu F, Liu X, Zhang J, et al. Clinical characteristics of 138 hospitalized patients with 2019 novel coronavirus–infected pneumonia in Wuhan, China. *JAMA.* (2020) 323:1061–9. doi: 10.1001/jama.2020.1585
14. Mao L, Wang M, Chen S, He Q, Chang J, Hong C, et al. Neurological manifestations of hospitalized patients with COVID-19 in Wuhan, China: a retrospective case series study. *BMJ.* (2020) 77:683–90. doi: 10.2139/ssrn.3544840
15. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet.* (2020) 395:497–506. doi: 10.1016/S0140-6736(20)30183-5
16. Chan JFW, Yuan S, Kok KH, To KKW, Chu H, Yang J, et al. A familial cluster of pneumonia associated with the 2019 novel coronavirus indicating person-to-person transmission: a study of a family cluster. *Lancet.* (2020) 395:514–23. doi: 10.1016/S0140-6736(20)30154-9
17. World Health Organization. *Q&A: Similarities and Differences – COVID-19 and Influenza.* (2020). Available online at: https://www.who.int/emergencies/diseases/novel-coronavirus-2019/question-and-answers-hub/q-a-detail/q-a-similarities-and-differences-covid-19-and-influenza (accessed June 22, 2020).
18. Tan W, Zhao X, Ma X, Wang W, Niu P, Xu W, et al. A novel coronavirus genome identified in a cluster of pneumonia cases—Wuhan, China 2019–2020. *China CDC Wkly.* (2020) 2:61–2. doi: 10.46234/ccdcw2020.017
19. Li Q, Guan X, Wu P, Wang X, Zhou L, Tong Y, et al. Early transmission dynamics in Wuhan, China, of novel coronavirus–infected pneumonia. *N Engl J Med.* (2020) 382:1199–207. doi: 10.1056/NEJMoa2001316
20. Lu R, Zhao X, Li J, Niu P, Yang B, Wu H, et al. Genomic characterisation and epidemiology of 2019 novel coronavirus: implications for virus origins and receptor binding. *Lancet.* (2020) 395:565–74. doi: 10.1016/S0140-6736(20)30251-8
21. Carlos WG, Dela Cruz CS, Cao B, Pasnick S, Jamil S. Novel Wuhan (2019-nCoV) coronavirus. *Am J Respir Crit Care Med.* (2020) 201:P7–8. doi: 10.1164/rccm.2014P7
22. Morawska L, Cao J. Airborne transmission of SARS-CoV-2: the world should face the reality. *Environ Int.* (2020) 139:105730. doi: 10.1016/j.envint.2020.105730
23. van Doremalen N, Bushmaker T, Morris DH, Holbrook MG, Gamble A, Williamson BN, et al. Aerosol and surface stability of SARS-CoV-2 as compared with SARS-CoV-1. *N Engl J Med.* (2020) 382:1564–7. doi: 10.1056/NEJMc2004973
24. He X, Lau EH, Wu P, Deng X, Wang J, Hao X, et al. Temporal dynamics in viral shedding and transmissibility of COVID-19. *Nat Med.* (2020) 26, 672–5. doi: 10.1038/s41591-020-0869-5
25. Walls AC, Park YJ, Tortorici MA, Wall A, McGuire AT, Veesler D. Structure, function, and antigenicity of the SARS-CoV-2 spike glycoprotein. *Cell.* (2020) 181:281–92.e6. doi: 10.1016/j.cell.2020.02.058
26. Chan JFW, Kok KH, Zhu Z, Chu H, To KKW, Yuan S, et al. Genomic characterization of the 2019 novel human-pathogenic coronavirus isolated from a patient with atypical pneumonia after visiting Wuhan. *Emerg Microbes Infect.* (2020) 9:221–36. doi: 10.1080/22221751.2020.1719902
27. McBride R, Van Zyl M, Fielding BC. The coronavirus nucleocapsid is a multifunctional protein. *Viruses.* (2014) 6:2991–3018. doi: 10.3390/v6082991
28. Neuman BW, Kiss G, Kunding AH, Bhella D, Baksh MF, Connelly S, et al. A structural analysis of M protein in coronavirus assembly and morphology. *J Struc Biol.* (2011) 174:11–22. doi: 10.1016/j.jsb.2010.11.021
29. Nieto-Torres JL, DeDiego ML, Verdia-Baguena C, Jimenez-Guardeno JM, Regla-Nava JA, Fernandez-Delgado R, et al. Severe acute respiratory syndrome coronavirus envelope protein ion channel activity promotes virus fitness and pathogenesis. *PLoS Pathogens.* (2014) 10:e1004077. doi: 10.1371/journal.ppat.1004077
30. Liu L, Wei Q, Alvarez X, Wang H, Du Y, Zhu H, et al. Epithelial cells lining salivary gland ducts are early target cells of severe acute respiratory syndrome coronavirus infection in the upper respiratory tracts of rhesus macaques. *J Virol.* (2011) 85:4025–30. doi: 10.1128/JVI.02292-10
31. Kuba K, Imai Y, Rao S, Gao H, Guo F, Guan B, et al. A crucial role of angiotensin converting enzyme 2 (ACE2) in SARS coronavirus-induced lung injury. *Nat Med.* (2005) 11:875–9. doi: 10.1038/nm1267
32. Chu H, Chan JFW, Yuen TTT, Shuai H, Yuan S, Wang Y, et al. Comparative tropism, replication kinetics, and cell damage profiling of SARS-CoV-2 and SARS-CoV with implications for clinical manifestations, transmissibility, and laboratory studies of COVID-19: an observational study. *Lancet Microbe.* (2020) 1:E14–23. doi: 10.1016/S2666-5247(20)30004-5
33. Jeffers SA, Tusell SM, Gillim-Ross L, Hemmila EM, Achenbach JE, Babcock GJ, et al. CD209L (L-SIGN) is a receptor for severe acute respiratory syndrome coronavirus. *Proc Natl Acad Sci USA.* (2004) 101:15748. doi: 10.1073/pnas.0403812101
34. Wang K, Chen W, Zhou YS, Lian JQ, Zhang Z, Du P, et al. SARS-CoV-2 invades host cells via a novel route: CD147-spike protein. *bioRxiv [Preprint].* (2020). doi: 10.1101/2020.03.14.988345
35. Bertram S, Glowacka I, Müller MA, Lavender H, Gnirss K, Nehlmeier I, et al. Cleavage and activation of the severe acute respiratory syndrome coronavirus spike protein by human airway trypsin-like protease. *J Virol.* (2011) 85:13363–72. doi: 10.1128/JVI.05300-11
36. Glowacka I, Bertram S, Müller MA, Allen P, Soilleux E, Pfefferle S, et al. Evidence that TMPRSS2 activates the severe acute respiratory syndrome coronavirus spike protein for membrane fusion and reduces viral control by the humoral immune response. *J Virol.* (2011) 85:4122–34. doi: 10.1128/JVI.02232-10
37. Siu YL, Teoh KT, Lo J, Chan CM, Kien F, Escriou N, et al. The M, E, and N structural proteins of the severe acute respiratory syndrome coronavirus are required for efficient assembly, trafficking, and release of virus-like particles. *J Virol.* (2008) 82:11318–30. doi: 10.1128/JVI.01052-08
38. Wu F, Zhao S, Yu B, Chen YM, Wang W, Song ZG, et al. A new coronavirus associated with human respiratory disease in China. *Nature.* (2020) 579:265–9. doi: 10.1038/s41586-020-2008-3
39. World Health Organization. *R&D Blueprint and COVID-19.* (2020). Available online at: https://www.who.int/teams/blueprint/covid-19 (accessed June 22, 2020).
40. Codagenix. *Codagenix and Serum Institute of India Initiate Co-Development of a Scalable, Live-Attenuated Vaccine Against the 2019 Novel Coronavirus, COVID-19.* (2020). Available online at: https://www.prnewswire.com/news-releases/codagenix-and-serum-institute-of-india-initiate-co-development-of-a-scalable-live-attenuated-vaccine-against-the-2019-novel-coronavirus-covid-19-301004654.html?tc=eml_cleartime (accessed June 22, 2020).
41. Cheung E. *China coronavirus: Hong Kong Researchers Have Already Developed Vaccine but Need Time to Test it, Expert Reveals: South China Morning Post.* (2020). Available online at: https://www.scmp.com/news/hong-kong/health-environment/article/3047956/china-coronavirus-hong-kong-researchers-have (accessed June 22, 2020).
42. Viroclinics Xplore. *International Partnership Progresses UQ COVID-19 Vaccine Project.* (2020). Available online at: https://www.uq.edu.au/news/article/2020/04/international-partnership-progresses-uq-covid-19-vaccine-project (accessed June 22, 2020).
43. GeoVax. *Geovax and Bravovax (Wuhan, China) to Collaborate on Development of Coronavirus Vaccine.* (2020). Available online at: https://www.geovax.com/news/geovax-and-bravovax-wuhan-china-to-collaborate-on- development-of-coronavirus-vaccine (accessed June 22, 2020).

44. Altimmune. *Altimmune Completes First Development Milestone Toward A Single-Dose Intranasal COVID-19 Vaccine.* (2020). Available online at: https://ir.altimmune.com/news-releases/news-release-details/altimmune-completes-first-development-milestone-toward-single (accessed June 22, 2020).
45. Clover Biopharmaceuticals. *Clover Initiates Development of Recombinant Subunit- Trimer Vaccine for Wuhan Coronavirus (2019-ncov).* (2020). Available online at: http://www.cloverbiopharma.com/index.php?m=content&c=index&a=show&catid=11&id=40 (accessed June 22, 2020).
46. CureVac. *About CureVac's Activities Regarding an mRNA Based Vaccine Against COVID-19.* (2020). Available online at: https://www.curevac.com/covid-19 (accessed June 22, 2020).
47. World health organization. *Novel Coronavirus – China.* (2020). Available online at: https://www.who.int/csr/don/12-january-2020-novel-coronavirus-china/en/ (accessed June 22, 2020).
48. Li F. Structure, function, and evolution of coronavirus spike proteins. *Ann Rev Virol.* (2016) 3:237–61. doi: 10.1146/annurev-virology-110615-042301
49. Li F, Li W, Farzan M, Harrison SC. Structure of SARS coronavirus spike receptor-binding domain complexed with receptor. *Science.* (2005) 309:1864–8. doi: 10.1126/science.1116480
50. Tai W, He L, Zhang X, Pu J, Voronin D, Jiang S, et al. Characterization of the receptor-binding domain (RBD) of 2019 novel coronavirus: implication for development of RBD protein as a viral attachment inhibitor and vaccine. *Cell Mol Immunol.* (2020) 17:613–20. doi: 10.1038/s41423-020-0400-4
51. Wrapp D, Wang N, Corbett KS, Goldsmith JA, Hsieh CL, Abiona O, et al. Cryo-EM structure of the 2019-nCoV spike in the prefusion conformation. *Science.* (2020) 367:1260–3. doi: 10.1126/science.abb2507
52. Ou X, Liu Y, Lei X, Li P, Mi D, Ren L, et al. Characterization of spike glycoprotein of SARS-CoV-2 on virus entry and its immune cross-reactivity with SARS-CoV. *Nat Commun.* (2020) 11:1620. doi: 10.1038/s41467-020-15562-9
53. Symvivo. *Covid-19 Program Vision.* (2019). Available online at: https://www.symvivo.com/covid-19 (accessed June 22, 2020).
54. Ahlberg V, Hjertner B, Wallgren P, Hellman S, Lövgren Bengtsson K, Fossum C. Innate immune responses induced by the saponin adjuvant matrix-M in specific pathogen free pigs. *Vet Res.* (2017) 48:30. doi: 10.1186/s13567-017-0437-2
55. Bengtsson KL, Song H, Stertman L, Liu Y, Flyer DC, Massare MJ, et al. Matrix-M adjuvant enhances antibody, cellular and protective immune responses of a zaire ebola/makona virus glycoprotein (GP) nanoparticle vaccine in mice. *Vaccine.* (2016) 34:1927–35. doi: 10.1016/j.vaccine.2016.02.033
56. Magnusson SE, Reimer JM, Karlsson KH, Lilja L, Bengtsson KL, Stertman L. Immune enhancing properties of the novel matrix-M^{TM} adjuvant leads to potentiated immune responses to an influenza vaccine in mice. *Vaccine.* (2013) 31:1725–33. doi: 10.1016/j.vaccine.2013.01.039
57. Novavax. *Novavax Identifies Coronavirus Vaccine Candidate; Accelerates Initiation of First-in-Human Trial to Mid-May Novavax.* (2020). Available online at: https://ir.novavax.com/news-releases/news-release-details/novavax-identifies-coronavirus-vaccine-candidate-accelerates (accessed June 22, 2020).
58. Kutzler MA, Weiner DB. DNA vaccines: ready for prime time? *Nat Rev Genet.* (2008) 9:776–88. doi: 10.1038/nrg2432
59. Gary EN, Weiner DB. DNA vaccines: prime time is now. *Curr Opin Immunol.* (2020) 65:21–7. doi: 10.1016/j.coi.2020.01.006
60. Smith TR, Patel A, Ramos S, Elwood D, Zhu X, Yan J, et al. Immunogenicity of a DNA vaccine candidate for COVID-19. *Nat Commun.* (2020) 11:2601. doi: 10.1038/s41467-020-16505-0
61. Muthumani K, Falzarano D, Reuschel EL, Tingey C, Flingai S, Villarreal DO, et al. A synthetic consensus anti–spike protein DNA vaccine induces protective immunity against middle east respiratory syndrome coronavirus in nonhuman primates. *Sci Transl Med.* (2015) 7:301ra132. doi: 10.1126/scitranslmed.aac7462
62. Kim YC, Dema B, Reyes-Sandoval A. COVID-19 vaccines: breaking record times to first-in-human trials. *NPJ Vaccin.* (2020) 5:34. doi: 10.1038/s41541-020-0188-3
63. Ewer K, Sebastian S, Spencer AJ, Gilbert S, Hill AV, Lambe T. Chimpanzee adenoviral vectors as vaccines for outbreak pathogens. *Hum Vaccin Immunother.* (2017) 13:3020–32. doi: 10.1080/21645515.2017.1383575
64. van Doremalen N, Haddock E, Feldmann F, Meade-White K, Bushmaker T, Fischer RJ, et al. A single dose of ChAdOx1 MERS provides protective immunity in rhesus macaques. *Science Adv.* (2020) 6:eaba8399. doi: 10.1101/2020.04.13.036293
65. Folegatti PM, Bittaye M, Flaxman A, Lopez FR, Bellamy D, Kupke A, et al. Safety and immunogenicity of a candidate middle east respiratory syndrome coronavirus viral-vectored vaccine: a dose-escalation, open-label, non-randomised, uncontrolled, phase 1 trial. *Lancet Infect Dis.* (2020) 20:816–82. doi: 10.1016/S1473-3099(20)30160-2
66. Oxford JIoUo. *A Study of a Candidate COVID-19 Vaccine (COV001).* (2020). Available online at: https://clinicaltrials.gov/ct2/show/study/NCT04324606 (accessed June 22, 2020).
67. University of Oxford OVG. *COVID-19 Vaccine Development.* (2020). Available online at: https://www.ovg.ox.ac.uk/news/covid-19-vaccine-development (acccessed June 22, 2020).
68. Prymula R, Siegrist CA, Chlibek R, Zemlickova H, Vackova M, Smetana J, et al. Effect of prophylactic paracetamol administration at time of vaccination on febrile reactions and antibody responses in children: two open-label, randomised controlled trials. *Lancet.* (2009) 374:1339–50. doi: 10.1016/S0140-6736(09)61208-3
69. Scheifele D, Ward B. Fever prophylaxis can reduce vaccine responses: a caution. *Paediatr Child Health.* (2018) 23:245–6. doi: 10.1093/pch/pxy011
70. Doedée AM, Boland GJ, Pennings JL, de Klerk A, Berbers GA, van der Klis FR, et al. Effects of prophylactic and therapeutic paracetamol treatment during vaccination on hepatitis B antibody levels in adults: two open-label, randomized controlled trials. *PloS ONE.* (2014) 9:e98175. doi: 10.1371/journal.pone.0098175
71. Espeseth AS, Cejas PJ, Citron MP, Wang D, DiStefano DJ, Callahan C, et al. Modified mRNA/lipid nanoparticle-based vaccines expressing respiratory syncytial virus F protein variants are immunogenic and protective in rodent models of RSV infection. *NPJ Vaccin.* (2020) 5:16. doi: 10.1038/s41541-020-0163-z
72. Pardi N, Hogan MJ, Weissman D. Recent advances in mRNA vaccine technology. *Curr Opin Immunol.* (2020) 65:14–20. doi: 10.1016/j.coi.2020.01.008
73. Moderna. *Moderna Ships mRNA Vaccine Against Novel Coronavirus (mRNA-1273) for Phase 1 Study.* (2020). Available online at: https://investors.modernatx.com/news-releases/news-release-details/moderna-ships-mrna-vaccine-against-novel-coronavirus-mrna-1273 (accessed June 22, 2020).
74. Moderna. *Safety and Immunogenicity Study of 2019-nCoV Vaccine (mRNA-1273) for Prophylaxis of SARS-CoV-2 Infection (COVID-19).* (2020). Available online at: https://www.clinicaltrials.gov/ct2/show/NCT04283461 (accessed Jun 22, 2020).
75. Norton TD, Miller EA. Recent advances in lentiviral vaccines for HIV-1 infection. *Front immunol.* (2016) 7:243. doi: 10.3389/fimmu.2016.00354
76. Shenzhen Geno-Immune Medical Institute. *Safety and Immunity of Covid-19 aAPC Vaccine.* (2020). Available online at: https://clinicaltrials.gov/ct2/show/NCT04299724 (accessed June 22, 2020).
77. Institute SGIM. *Immunity and Safety of Covid-19 Synthetic Minigene Vaccine.* (2020). Available online at: https://clinicaltrials.gov/ct2/show/NCT04276896 (accessed June 22, 2020).
78. Pfizer. *Pfizer and Biontech Dose First Participants in the U.S. as Part of Global Covid-19 Mrna Vaccine Development Program.* (2020). Available online at: https://www.pfizer.com/news/press-release/press-release-detail/pfizer_and_biontech_dose_first_participants_in_the_u_s_as_part_of_global_covid_19_mrna_vaccine_development_program (accessed June 22, 2020).
79. Thanh Le T, Andreadakis Z, Kumar A, Gómez Román R, Tollefsen S, Saville M, et al. The COVID-19 vaccine development landscape. *Nat Rev Drug Discov.* (2020) 19:305–6. doi: 10.1038/d41573-020-00073-5
80. Zhu FC, Li YH, Guan XH, Hou LH, Wang WJ, Li JX, et al. Safety, tolerability, and immunogenicity of a recombinant adenovirus type-5 vectored COVID-19 vaccine: a dose-escalation, open-label, non-randomised, first-in-human trial. *Lancet.* (2020) 39:1845–54. doi: 10.1016/S0140-6736(20)31208-3
81. Fausther-Bovendo H, Kobinger GP. Pre-existing immunity against Ad vectors: humoral, cellular, and innate response, what's important? *Hum Vaccin Immunother.* (2014) 10:2875–84. doi: 10.4161/hv.29594

82. Curtis N, Sparrow A, Ghebreyesus TA, Netea MG. Considering BCG vaccination to reduce the impact of COVID-19. *Lancet.* (2020) 395:1545–6. doi: 10.1016/S0140-6736(20)31025-4
83. Soliman R, Brassey J, Plüddemann A. *Does BCG Vaccination Protect Against Acute Respiratory Infections and COVID-19? A Rapid Review of Current Evidence*. Oxford: The Centre for Evidence-Based Medicine (2020).
84. Arts R, Moorlag S, Novakovic B, Li Y, Wang SY, Oosting M, et al. BCG vaccination protects against experimental viral infection in humans through the induction of cytokines associated with trained immunity. *Cell Host Microbe.* (2018) 23:89–100.e5. doi: 10.1016/j.chom.2017.12.010
85. Old LJ, Benacerraf B, Clarke DA, Carswell EA, Stockert E. The role of the reticuloendothelial system in the host reaction to neoplasia. *Cancer Res.* (1961) 21:1281–300.
86. Floc'h F, Werner G, editor. *Increased Resistance to Virus Infections of Mice Inoculated With BCG (Bacillus calmette-guérin)*. In: Annales d'immunologie (1976).
87. Izmirly A, Alturki SO, Alturki SO, Connors J, Haddad E. Challenges in dengue vaccines development: pre-existing infections and cross-reactivity. *Front. Immunol.* (2020) 11:1055. doi: 10.3389/fimmu.2020.01055
88. Takada A, Ebihara H, Feldmann H, Geisbert TW, Kawaoka Y. Epitopes required for antibody-dependent enhancement of ebola virus infection. *J infect Dis.* (2007) 196(Suppl. 2):S347–56. doi: 10.1086/520581
89. Kuzmina NA, Younan P, Gilchuk P, Santos RI, Flyak AI, Ilinykh PA, et al. Antibody-dependent enhancement of ebola virus infection by human antibodies isolated from survivors. *Cell Rep.* (2018) 24:1802–15.e5. doi: 10.1016/j.celrep.2018.07.035
90. Tay MZ, Liu P, Williams LD, McRaven MD, Sawant S, Gurley TC, et al. Antibody-mediated internalization of infectious HIV-1 virions differs among antibody isotypes and subclasses. *PLoS Pathogens.* (2016) 12:e1005817. doi: 10.1371/journal.ppat.1005817
91. Takada A, Kawaoka Y. Antibody-dependent enhancement of viral infection: molecular mechanisms and in vivo implications. *Rev Med Virol.* (2003) 13:387–98. doi: 10.1002/rmv.405
92. Sridhar S, Luedtke A, Langevin E, Zhu M, Bonaparte M, Machabert T, et al. Effect of dengue serostatus on dengue vaccine safety and efficacy. *N Engl J Med.* (2018) 379:327–40. doi: 10.1056/NEJMoa1800820
93. Wan Y, Shang J, Sun S, Tai W, Chen J, Geng Q, et al. Molecular mechanism for antibody-dependent enhancement of coronavirus entry. *J Virol.* (2020) 94:e02015–19. doi: 10.1128/JVI.02015-19
94. Ho MS, Chen WJ, Chen HY, Lin SF, Wang MC, Di J, et al. Neutralizing antibody response and SARS severity. *Emerg Infect Dis.* (2005) 11:1730. doi: 10.3201/eid1111.040659
95. Tetro JA. Is COVID-19 receiving ADE from other coronaviruses? *Microbes Infect.* (2020) 22:72–3. doi: 10.1016/j.micinf.2020.02.006
96. Iwasaki A, Yang Y. The potential danger of suboptimal antibody responses in COVID-19. *Nat Rev Immunol.* (2020) 20:339–41. doi: 10.1038/s41577-020-0321-6
97. Yang ZY, Werner HC, Kong WP, Leung K, Traggiai E, Lanzavecchia A, et al. Evasion of antibody neutralization in emerging severe acute respiratory syndrome coronaviruses. *Proc Natl Acad Sci USA.* (2005) 102:797–801. doi: 10.1073/pnas.0409065102

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cell and Developmental Biology

ORIGINAL RESEARCH
published: 20 August 2020
doi: 10.3389/fcell.2020.00831

SARS-CoV-2 Codon Usage Bias Downregulates Host Expressed Genes With Similar Codon Usage

Andres Mariano Alonso[1,2] *and Luis Diambra*[2,3]*

[1] InTech, Universidad Nacional de San Martin, Chascomús, Argentina, [2] CONICET, Chascomús, Argentina, [3] CREG, Universidad Nacional de La Plata, La Plata, Argentina

Severe acute respiratory syndrome has spread quickly throughout the world and was declared a pandemic by the World Health Organization (WHO). The pathogenic agent is a new coronavirus (SARS-CoV-2) that infects pulmonary cells with great effectiveness. In this study we focus on the codon composition for the viral protein synthesis and its relationship with the protein synthesis of the host. Our analysis reveals that SARS-CoV-2 preferred codons have poor representation of G or C nucleotides in the third position, a characteristic which could result in an unbalance in the tRNAs pools of the infected cells with serious implications in host protein synthesis. By integrating this observation with proteomic data from infected cells, we observe a reduced translation rate of host proteins associated with highly expressed genes and that they share the codon usage bias of the virus. The functional analysis of these genes suggests that this mechanism of epistasis can contribute to understanding how this virus evades the immune response and the etiology of some deleterious collateral effect as a result of the viral replication. In this manner, our finding contributes to the understanding of the SARS-CoV-2 pathogeny and could be useful for the design of a vaccine based on the live attenuated strategy.

Keywords: SARS-CoV-2, codon usage bias, codon optimality, translational control, pathogeny, vaccine design

OPEN ACCESS

Edited by:
Vasu D. Appanna, Laurentian University, Canada

Reviewed by:
Roosevelt Alves DaSilva, Universidade Federal de Goiás, Brazil
Sayed-Amir Marashi, University of Tehran, Iran

***Correspondence:**
Luis Diambra
ldiambra@gmail.com

Specialty section:
This article was submitted to Molecular Medicine, a section of the journal Frontiers in Cell and Developmental Biology

Received: *01 May 2020*
Accepted: *04 August 2020*
Published: *20 August 2020*

Citation:
Alonso AM and Diambra L (2020) SARS-CoV-2 Codon Usage Bias Downregulates Host Expressed Genes With Similar Codon Usage. Front. Cell Dev. Biol. 8:831. doi: 10.3389/fcell.2020.00831

1. INTRODUCTION

The new SARS-CoV-2 coronavirus is the causative agent of the current pandemic of COVID-19. This highly pathogenic virus has quickly become the latest threat to modern life. From the end of 2019 and up to the redaction of this paper, this virus has infected over 17 million people, leading to mild symptoms of fever and to lung function reduction, severe acute respiratory syndrome (SARS), and even death. The current global death toll stands at 670,000; however, we are still far away from determining the final mortality figure. In the absence of vaccines or effective antiviral treatments against SARS-CoV-2, it is important to understand how this virus appropriates the host translation apparatus and subverts the immune defenses of infected cells. This can be the first step in the development of novel therapeutics.

As intracellular parasites, virus replication depends on the translational machinery of their cellular hosts to translate viral transcripts. Thus, virus replication requires ribosomes, tRNA, and translation factors from the cell host. On the other hand, codon usage bias is a feature of natural selection and affects the genomes of all domains of life. It is known that more frequently used codons are used for coding highly abundant proteins (Pan et al., 1998; Dana and Tuller, 2014; Quax et al., 2015; Diambra, 2017). Virus genomes also have preferences in the codon usage, but, in this

case, the bias is constrained by the host translational machinery (Shackelton et al., 2006). The effects of codon composition of a transcript on its translation have been reported in literature (Gingold and Pilpel, 2011; Plotkin and Kudla, 2011; Shah and Gilchrist, 2011; McCarthy et al., 2017) and is considered an important determinant of gene expression (Zhou et al., 2009; Tuller et al., 2010a,b). However, the codon usage of a gene can also affect the translation of other genes (Frumkin et al., 2018). In fact, the virus replication demands not only ribosomes but also a lot tRNA resources for the codons highest in demand (Chen et al., 2020). The consumption of specific tRNAs for the virus replication could thus be an alternative to controlling host protein synthesis machinery as well as generating deleterious collateral effects on the function of the host cells. Recent evidence supports the idea that high codon usage similarity between virus and host can lead to a deleterious effect on the host (Chen et al., 2020). Furthermore, Chen et al. (2020) have shown that codon composition of highly expressed viral genes regulates the tRNA availability, affecting the decoding time of codons in the infected cells. In this manner the viral infection can convert abundant codons into scarce codons, reshaping the human codon optimality pattern. In this sense, it has been proposed that dysregulation of the tRNA pool can lead to disease (Dhindsa et al., 2020). In fact, there is growing evidence supporting that synonymous variations in a coding sequence can affect its folding and/or resulting expression level (Tsai et al., 2008; Hunt et al., 2014; Buhr et al., 2016; Kirchner et al., 2017; von Herrmann et al., 2018; Dhindsa et al., 2020; Kim et al., 2020).

It is known that coronavirus genomes are poor in GC content ($\sim$ 40%) and that there exist a preferential use of A-ended or U-ended codons in these genomes (Gu et al., 2004; Kandeel et al., 2020). The last bias is usually characterized in the literature as the GC3 content. Indeed, the analysis performed by Gu et al. (2004) suggests that this compositional constraint is a major determinant to synonymous codon usage. Here, we study recent proteomic data from SARS-CoV-2 infected cells (Bojkova et al., 2020) from a novel point of view. By considering the codon usage of the virus ORFeome, we characterize a set of genes whose expression could be affected by the massive demand of the tRNA which implies the virus replication. We find that those host genes encoding proteins with similar codons to the virus ORFeome have a lower translational rate. Extrapolating this finding to highly expressed genes in lung, we find a small set of genes that can be downregulated. These genes are involved in translation, immune systems, and cell calcification, to name a few, and their roles in the SARS-CoV-2 cell pathogenesis should be the target of further studies.

2. MATERIALS AND METHODS

The coding sequences associated with the genome of SARS-CoV and SARS-CoV-2 were obtained from the NCBI (NC_004718.3 and NC_045512.2, respectively). Highly expressed genes in the lung and arterial tissues were retrieved from the GTEx portal (gtexportal.org). Proteomic and translatomic data from infected CACO-2 cells were retrieved from Supplementary Material of Bojkova et al. (2020), which are publicly available (ProteomeXchange repository, ID = PXD017710). These data contain protein levels from 6,381 proteins and translational rate from 2,715 proteins, and the proteomic information is consequently not available for many proteins.

Given the coding sequence s, we compute the codon usage frequency as $f_s(c) = \frac{N_c}{L_s}$, where L_s is total number of codons in the sequence s, and N_c is the number of times that codon c is present in s. In a similar manner, we define codon usage frequency relative to the viral ORFome: $f_O(c) = \frac{N_c}{L_O}$, where L_O is the total number of codons in the ORFome, and N_c is the number of occurrences observed codon c in the ORFome. Thus, $f_s(c)$ and $f_O(c)$ are vector of 64 elements. To compute the CCorr of a given sequence s with viral ORFome, we consider the Pearson's correlation coefficient between the vector $f_s(c)$ and $f_O(c)$:

$$\mathrm{CCorr} = \frac{\sum_c \left(\mathrm{f_s}\,(\mathrm{c}) - \overline{\mathrm{f_s}}\right).\left(\mathrm{f_O}\,(\mathrm{c}) - \overline{\mathrm{f_O}}\right)}{\sqrt{\sum_c \left(\mathrm{f_s}\,(\mathrm{c}) - \overline{\mathrm{f_s}}\right)^2}.\sqrt{\sum_c \left(\mathrm{f_O}\,(\mathrm{c}) - \overline{\mathrm{f_O}}\right)^2}} \quad (1)$$

3. RESULTS

We study the codon composition of two complete coronavirus genomes by measuring the percentage of G and C nucleotides at the wobble position of the codons (GC3 content). **Figure 1A** depicts the GC3 content of each annotated coding sequence of the SARS-CoV and SARS-CoV-2. This analysis reveals that, in agreement with Gu et al. (2004) and Kandeel et al. (2020), codons preferentially used by these viruses are associated with a lower GC3 content than that expected from the random use of nucleotides. In particular, the ORFs corresponding to the proteins ORF1ab, ORF1a, surface, ORF6, ORF7a, and ORF8 exhibit a very low content of GC3. Specifically, the last two proteins are highly expressed at 10 h post-infection (PI), as can be seen in **Figure 1B**.

As was highlighted previously, this high expression of viral proteins could lead to an imbalance in the tRNA pool needed for the normal synthesis of the proteins of the host cell. Of course, as tRNA pools depend on the cell types, the postulated imbalance could be a tissue-dependent feature. To check this hypothesis, we make use of the recently available data about the proteome profile and translational rate in SARS-CoV-2-infected cells (Bojkova et al., 2020). In this study, a CACO-2 cell line was infected with SARS-CoV-2 and mocked, and the last one was used as the control. From this proteome, we selected the coding sequences corresponding to the 100 most abundant proteins in the mock infected cells at 10 h PI. We then computed the codon correlation (CCorr) between the codon usage of each one of these sequences and the codon usage of all coding sequences in SARS-CoV-2. **Figure 2** depicts a scatter plot of the translational rate of these genes from SARS-CoV-2-infected cells vs. the corresponding CCorr (red dots). The black line is the adjusted linear model, which shows a significant and negative correlation between the translation rate and the codon composition of each sequence. This means that the translational rate of coding

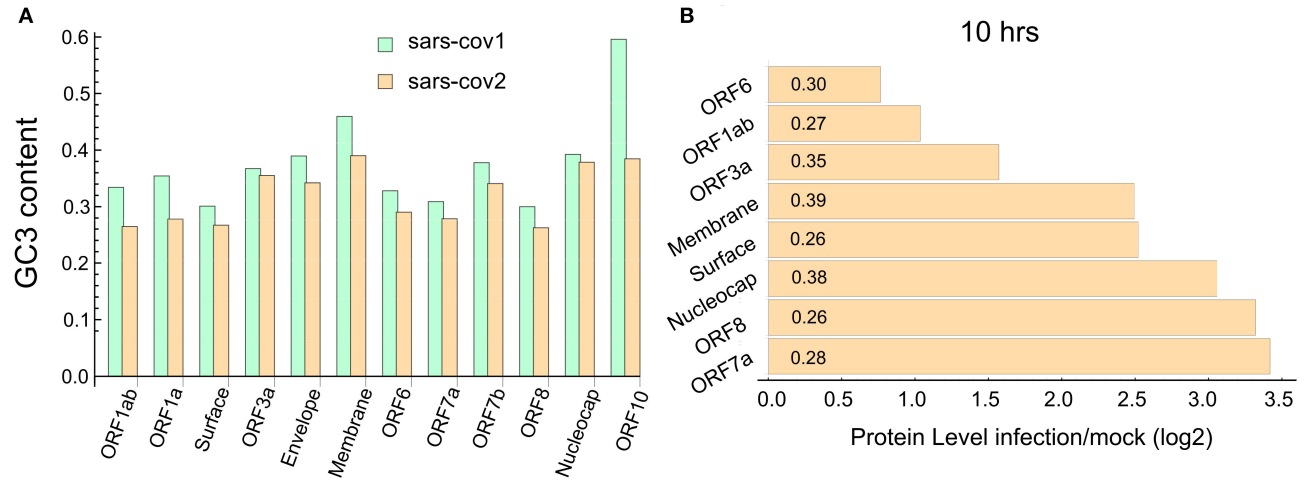


FIGURE 1 | SARS-CoV-2 ORFome have lower content of GC3. **(A)** Bar chart comparing the G and C nucleotides on the third nucleotide (GC3) at every codon in the ORFeome of SARS-CoV and SARS-CoV-2. **(B)** Bar chart showing the proteins level of nine viral proteins at 10 h post-infection (data obtained from Bojkova et al., 2020). The GC3 content for each viral ORF is showed in the corresponding bar.

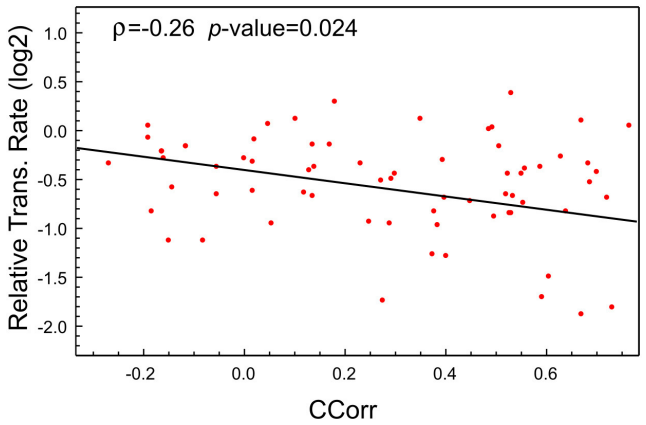


FIGURE 2 | The translational rate of genes with high CCorr in SARS-CoV-2-infected cells is lower than the mock-infected cells. Scatter plot showing negative correlation between translational rate of the 100 most expressed proteins in SARS-CoV-2 infected CACO-2 cells vs. virus codon usage.

sequence in SARS-CoV-2-infected cells is lower than in mock-infected cells. Although the correlation ($\rho = -0.26$) with codon composition is not high, reflecting the fact that other factors could be regulating the translation rate, it is significant at a value of 0.05 (p-value $= 0.024 < 0.05$).

This analysis suggests then that the codon composition of highly expressed host-genes is a determinant of its own translation rate and that viral replication induces a particular case of epistasis, which could affect host cell proteostasis. Consequently, we find it relevant to extrapolate this phenomenon to cells affected by SARS-CoV-2. In this sense, the ACE2 receptor has been identified as the SARS-CoV-2 cell entry. Even though ACE2 transcript is present in almost all organs, the surface expression of ACE2 protein is present in lung alveolar epithelial cells and arterial and venous endothelial cells (Hamming et al., 2004). For that reason, we elected to analyze the most expressed genes in lung and arterial tissues extracted from the GTEx database, two main targets of the SARS-CoV-2 infection. To this end, we select the 100 most expressed genes in these tissues and compute the CCorr for each sequence. **Figure 3A** shows that the codon composition profile of the most expressed genes from both lung and CACO-2 cells types are different, it is evident that the lung cells share fewer codons in common with the SARS-CoV-2 than the CACO-2 cells. On the other hand, the codon composition profile of the highly expressed genes in arterial tissue are quite similar to the genes associated with the lung (**Figure 3B**). In fact, they share 60% of these highly expressed genes (**Figure 3C**). Consequently, the tRNAs pool used for the virus could be scarcer both in lung and arterial cells than in the CACO-2 cells.

We search for those genes, highly expressed in the lung tissue, which could be affected by the depletion of the tRNAs consumed by virus replication. They are those whose codon composition is similar to SARS-CoV-2 (CCorr > 0.25); these are listed in **Table 1**. Before analyzing these 27 genes of interest, we compared the translation rate of these genes with the translation rate associated with highly expressed genes, but which differs in their codon usage bias (CCorr < -0.075). In this sense, we used the Mann-Whitney U test for median differences of independent samples to analyze the difference in translational rate between these two groups of genes. This test identified significant differences between these groups (p-value $= 0.0007$), as it can be seen in **Figure 3D**. We want to remark that, since there is still no available data on translational rate from lung cells, the last analysis was performed by using translational rate data available from CACO-2 cells, although we know that this data would be underestimating the difference between these two groups of genes.

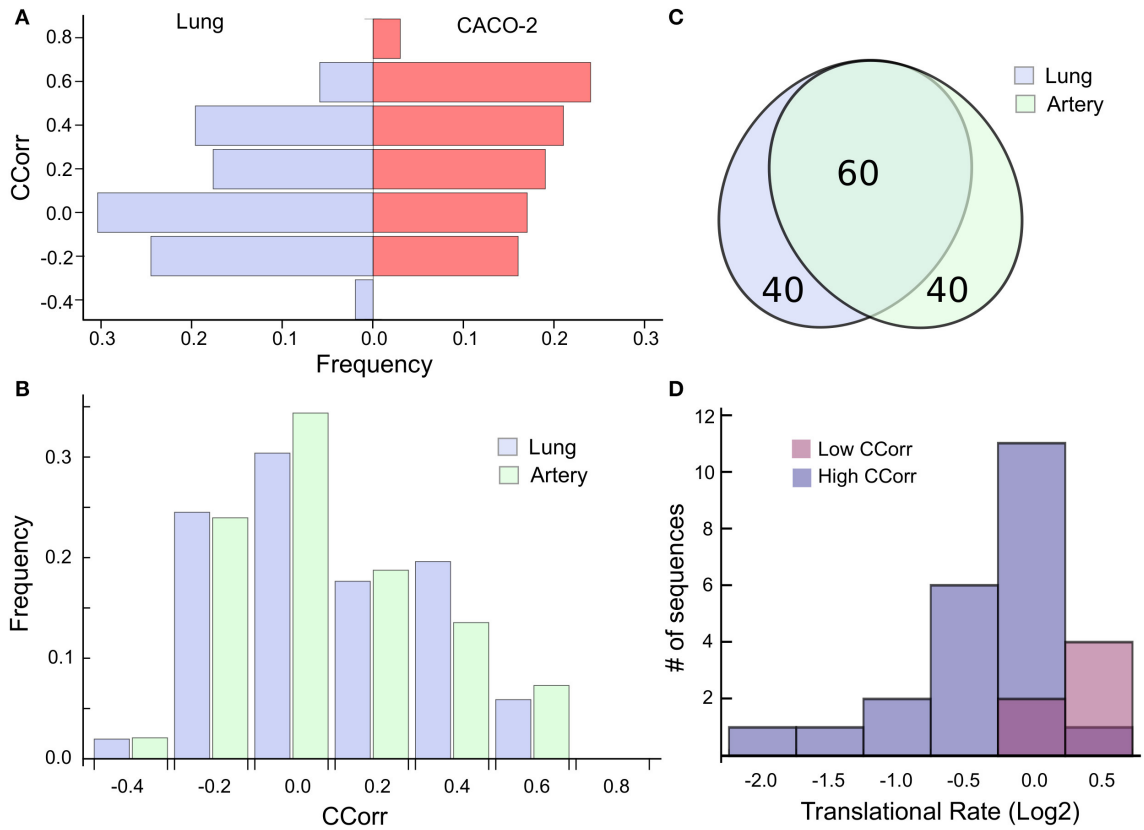


FIGURE 3 | High codon correlation between SARS-CoV-2 and lung cells is associated with a translational rate decay over specific transcripts. **(A)** Frequency distribution of CCorr on the 100 highest expressed genes in lung cells and CACO-2 cells. **(B)** Comparison between the CCorr frequency distributions obtained from the 100 highest expressed genes in lung and arterial tissues. **(C)** The tissues of the lungs and arteries share most of the highly expressed genes. **(D)** Histogram of the translational rate of 100 highest expressed genes in lung cells. Transcripts with high and low codon correlation (CCorr) with SARS-CoV-2 are highlighted.

Furthermore, we performed a GO term enrichment analysis over the 27 genes listed in **Table 1**; their translation rates are decreased by means of the Enrichr online software (Chen et al., 2013). **Figure 4** illustrates the main enrichment pathways. This analysis reveals that codon usage could promote extensive changes in the translation machinery of the host in agreement with previous report in CACO-2 cells (Bojkova et al., 2020). It is known that when canonical translation is impaired, as part of the host defense program, specific 40S ribosomal subunits are needed to support uncapped viral mRNA translation (Kwan and Thompson, 2019).

Our list of genes from lung is enriched with several ribosomal proteins that constitute both 40S (RPS6, RPS3A, RPS7, RPSA, RPS25, RPS13, RPS12, and RPS27A) and 60S (RPL4, RPL5, RPL21, RPLP1, RPL34, RPL9, RPL24, and RPL17) ribosomal subunits. Many of them also appear in the set of genes derived from arterial tissue. All of them belong to the nonsense-mediated decay pathway that control the mRNAs with abnormal termination. The termination codon can be recognized if the 3′ untranslated region is short or if it does not have an exon junction complex downstream of the termination codon (Nicholson et al., 2010). These genes are also part of the process of SRP-dependent co-translational protein destined for the endoplasmic reticulum.

It is known that these proteins are involved in translation particularly the gene EEF1A1, an isoform of the alpha subunit of the elongation factor-1 expressed in lungs and arteries, which is responsible for the GTP-dependent binding of aminoacyl-tRNA to the ribosome. Beyond its function in translation, EEF1A1 takes part in the innate immune system by activating directly the transcription of IFN-gamma (Maruyama et al., 2007). IFN-gamma activates immune cells, such as macrophages and natural killer cells, and stimulates the major histocompatibility complex (MHC) II-dependent presentation of antigens to the immune system (Schroder et al., 2004). This process could be downregulated due to a decrease in the translation rate of EEF1A1. Furthermore, B2M, another gene in the list of decreased translational rate, encodes one of the proteins that conform MHC class I found on the cell surface of all nucleated cells (Bernier, 1980). Together with TXNIP, RPS3A, and RPS27A, it is involved in the antigen processing and presentation. Moreover, this gene, along with S100A11, HSP90AB1, and also EEF1A1, regulates the exocytosis of granules containing inflammatory mediators in neutrophils (Lee et al., 2005). All these genes, with the exception of TXNIP and S100A11, are also highly expressed in the arterial tissue. We highlight the presence in our analysis of the HSP90AB1, a known chaperone that facilitates the

TABLE 1 | Set of most expressed genes in lung (GTEx) and positive correlation with virus codon usage.

Gene name	Codon corr	T. rate 6 h	T. rate 10 h
RPS3A	0.582	0.686	0.980
RPL5	0.559	0.585	0.271
RPL9	0.546	0.723	0.738
SPARCL1	0.536	NA	NA
EEF1A1	0.531	0.948	0.627
RPS12	0.443	0.155	0.649
RPL17	0.438	NA	0.372
TPT1	0.415	1.048	0.981
RPL21	0.411	NA	NA
RPS6	0.398	1.206	0.590
RPL34	0.376	0.981	0.727
RPS13	0.372	1.386	0.209
RPL4	0.370	0.919	0.799
RPS27A	0.365	NA	NA
RPSA	0.352	0.902	0.643
SAT1	0.350	NA	NA
A2M	0.340	NA	NA
TXNIP	0.317	NA	NA
RPS25	0.315	0.728	0.753
FN1	0.308	NA	NA
RPL24	0.306	0.576	0.890
RPLP1	0.303	NA	NA
RPS7	0.297	0.965	1.036
HSP90AB1	0.296	0.989	0.738
B2M	0.277	0.926	0.825
S100A11	0.273	1.620	0.520
MGP	0.272	NA	NA

col 1: gene name, col 2: CCorr, cols 3 and 4: translational rate, infected CACO-2 cells, measured at 6 and 10 h PI, respectively. Translational rates are relative to mock cells.

maturation of a wide range of proteins and its attenuation has been related to idiopathic pulmonary fibrosis and cystic fibrosis (Haase and Fitze, 2015; Wang and Ni, 2016). Our analysis also shows a translation decrease in A2M, a plasmatic protein highly expressed in lung and artery that inhibits a broad spectrum of proteases, including trypsin, thrombin, and collagenase (Cater et al., 2019). A recent study confirms our prediction, A2M level in sera from COVID-19 patients is significantly lower than in healthy subjects (Shen et al., 2020). Furthermore, A2M has a key role in regulating inflammatory processes because is able to bind to proinflammatory ligands (Feige et al., 1996). This is particularly relevant in the context of the COVID-19-derived cytokine storm.

In addition, we have also identified two genes (SAT1 and MGP) belonging to the pathway of endothelial cell calcification regulated by NOTCH1 (White et al., 2015). This finding acquires special relevance in the context of the acute lung injury (ALI) observed in many infected patients (Huang et al., 2020). The first gene SAT1 catalyzes the acetylation of polyamines (spermidine and spermine) and carries it out of the cell. The polyamine excess is a prominent source of oxidative stress that can increase inflammatory response (Hussain et al., 2017). Polyamines have also been connected with the immune system (Pérez-Cano et al., 2003). On the other hand, the MGP gene encodes the matrix gla protein, which is also highly expressed in all vasculatures. Recent studies suggest that MGP downregulates the tissue calcification by sequestering bone morphogenetic proteins (White et al., 2015). Mutations in this gene cause Keutel syndrome, which is characterized by peripheral pulmonary stenosis, abnormal cartilage calcification, and skin rashes (Munroe et al., 1999).

Another interesting protein predicted to be downregulated during the infection in lung and arterial tissues is SPARC-like 1 (SPARCL-1), also known as Hevin, commonly associated with regulation of cell migration and modulation of extracellular matrix proteins (Girard and Springer, 1996), and it has been shown to be involved in lymphocyte transendothelial migration through high endothelial venules (Girard and Springer, 1995). In this context, it is important to mention that clinical studies over patients that suffer several cases of COVID-19 document a dysregulation of immune response related particularly to a lower lymphocytes count (Huang et al., 2020). In addition, our analysis reveals a translational rate decay of fibronectin (FN1), a master organizer of extracellular matrices that mediates cellular interactions playing important roles in cell adhesion, hemostasis, and thrombosis (Pankov and Yamada, 2002; Wang and Ni, 2016). This prediction is in agreement with previous data showing that cells infected with SARS-CoV undergo downregulation in fibronectin expression (Surjit et al., 2004).

4. DISCUSSION

The viral infection of human cells triggers an ensemble of host processes based on the interferons that interfere with viral replication. These processes have co-evolved with the viral response to the host defense, and the virus counteracts these processes through a diversity of immuno-modulatory mechanisms. For example, NS1 protein plays a central role in the influenza infection by suppressing the host IFNs response. Recent results suggest that NS1 protein can hamper the host gene expression at the translational level by obstructing the mRNA entrance tunnel of ribosomes (Thoms et al., 2020). Furthermore, the N protein of porcine reproductive and respiratory syndrome virus impairs the IFN transcription by acting over the TXK, which together with the EEF1A1 and PARP1 form the trimolecular complex that binds to the IFN-γ gene promoter (Maruyama et al., 2007; Kenney and Meng, 2015). Hemagglutinin of IAVs has been shown to facilitate IFNAR ubiquitination and degradation, reducing the levels of IFNAR, and thus suppressing the expression of IFN-stimulated antiviral proteins (Xia et al., 2016). These examples illustrate the action of viral dedicated factors that downregulate the transcription of IFNs. Up to the present, no dedicated factor with analog function has been identified in SARS-CoV and SARS-CoV-2. However, a recent report found a significant lack of IFN type I and III at the transcriptional level in human alveolar adenocarcinoma cells (Blanco-Melo et al., 2020). On the other hand, a marked upregulation of inflammatory

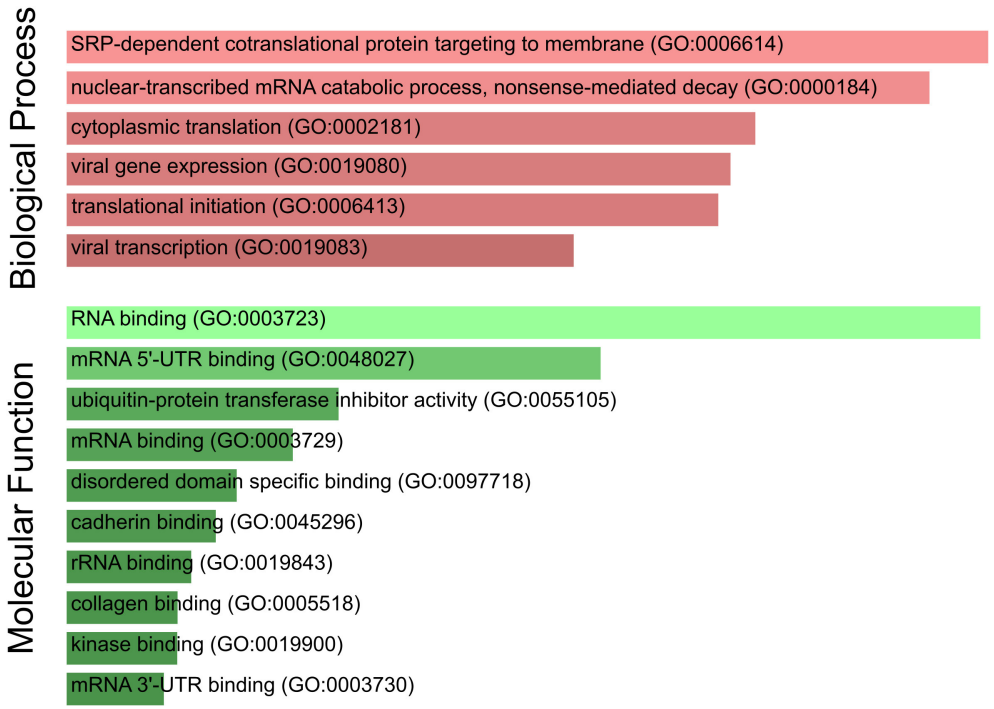


FIGURE 4 | SARS-CoV-2 codon usage may have an impact on host translational machinery. GO enrichment analysis performed over genes of interest. The most enriched biological processes and molecular functions are highlighted. Both size and color shade of bars represent the level of significance.

mediators at the protein level (CXCL10, CCL2, IFN-α, and γ) has been observed in patients with SARS-CoV without a significant amount of specific antibodies (Cameron et al., 2007). Several cases of absence of protective immunity due to previous infection seem to indicate a similar landscape for COVID-19. Until now, the manner in which some patients fail to develop adaptive immunity is yet to be elucidated. As mentioned in the Results section, the decreased translation of B2M could be related to this last observation since is a crucial factor for the stable presentation of antigens derived from virus or tumor proteins; these antigens are recognized by cytotoxic T cells that eventually eliminate the target cell stimulating apoptosis to prevent systemic dissemination of the disease (Hulpke and Tampé, 2013).

MGP is also expressed at high levels in heart, kidney, and lung which is particularly interesting in the context of several comorbidities and collateral effects observed in the COVID-19 patients (Nikolich-Zugich et al., 2020). For example, skin rashes were recently reported as a new symptom of COVID-19 and the authors postulate that recognizing rashes is important to identify new and earlier COVID-19, cases (Bataille et al., 2020). In this context, we highlight that permanent skin rashes were reported as a characteristic of the Keutel syndrome, and mutations in the MGP gene were reported as a crucial factor in this syndrome (Munroe et al., 1999; Khosroshahi et al., 2014). In this manner, our results are related to these observations.

Summing up, if the depletion of a selected set of tRNA, induced by virus replication, affects the expression level or the co-translation folding of these proteins, one could expect the emergence of several systemic disorders.

5. CONCLUSION

Codon usage bias is thought to have significant effects on translation rate, where rare codons are assumed to be translated more slowly than common codons (Piovesan et al., 2013). It is assumed that rare and common codons are defined by usage rates of highly expressed genes. However, whether the codon composition of viral ORFome can affect the translation rate of host genes has not been thoroughly explored yet. Here, we have shown that the synthesis of the proteins associated with highly expressed genes, and with similar codon usage to the one of the virus, appears to be downregulated. This finding is in agreement with recent observations in totivirus-infected yeast (Chen et al., 2020). Following this idea, we determined which genes in lung could be affected by the viral replication. A functional analysis of these genes reveals that they could be related to collateral effects observed in COVID-19 patients (Huang et al., 2020). Further studies are mandatory to corroborate or discard the putative relationship established here.

One of the main obstacles in the recent development of vaccines has been the finding of increased infectivity observed to occur after immunizations with whole virus vaccines or complete spike protein vaccines. This phenomenon has been observed both in vaccines against SARS coronavirus and in respiratory

syncytial virus. However, just as the virus regulates the translation of the host by its codon usage, the biotechnological manipulation of the frequency of codons could be used to design attenuated viruses. In this sense, other vaccine strategy has been recently assayed, focusing on altering the codon-pair usage without affecting protein sequence. This codon deoptimization strategy has reduced virus replication (Coleman et al., 2008; Le Nouen et al., 2014). We believe that our results shed light on how codon use could affect virus attenuation and would help decrease the damaging side effect, providing an exciting opportunity for live-attenuated vaccine development.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: http://corona.papers.biochem2.com; http://proteomecentral.proteomexchange.org/cgi/GetDataset?ID=PXD017710.

AUTHOR CONTRIBUTIONS

LD designed the experiments. AA prepared the data. LD and AA the analyzed the data and wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

AA was a post-doctoral fellow of the CONICET (Argentina), and LD was a research member of the CONICET (Argentina).

ACKNOWLEDGMENTS

We would like to thank Nara Guisoni, Alejandra Carrea, Diego Bustos, and Veronica Cóceres for their insightful suggestions and discussion. We were thankful to Catalina Trousdell for her judicious comments on the manuscript.

REFERENCES

Bataille, V., Visconti, A., Rossi, N., Murray, B., Bournot, A., Wolf, J., et al. (2020). Diagnostic value of skin manifestation of SARS-CoV-2 infection. *medRxiv*. doi: 10.1101/2020.07.10.20150656

Bernier, G. M. (1980). β2-microglobulin: structure, function and significance. *Vox Sanguinis* 38, 323–327. doi: 10.1159/000467018

Blanco-Melo, D., Nilsson-Payant, B. E., Liu, W. C., Uhl, S., Hoagland, D., Møller, R., et al. (2020). Imbalanced host response to SARS-CoV-2 drives development of COVID-19. *Cell* 318, E736–E741. doi: 10.1016/j.cell.2020.04.026

Bojkova, D., Klann, K., Koch, B., Widera, M., Krause, D., Ciesek, S., et al. (2020). Proteomics of SARS-CoV-2-infected host cells reveals therapy targets. *Nature* 583, 469–472. doi: 10.1038/s41586-020-2332-7

Buhr, F., Jha, S., Thommen, M., Mittelstaet, J., Kutz, F., Schwalbe, H., et al. (2016). Synonymous codons direct cotranslational folding toward different protein conformations. *Mol. Cell* 61, 341–351. doi: 10.1016/j.molcel.2016.01.008

Cameron, M. J., Ran, L., Xu, L., Danesh, A., Bermejo-Martin, J. F., Cameron, C. M., et al. (2007). Interferon-mediated immunopathological events are associated with atypical innate and adaptive immune responses in patients with severe acute respiratory syndrome. *J. Virol.* 81, 8692–8706. doi: 10.1128/JVI.00527-07

Cater, J. H., Wilson, M. R., and Wyatt, A. R. (2019). Alpha-2-macroglobulin, a hypochlorite-regulated chaperone and immune system modulator. *Oxidat. Med. Cell. Longev.* 2019:5410657. doi: 10.1155/2019/5410657

Chen, E. Y., Tan, C. M., Kou, Y., Duan, Q., Wang, Z., Meirelles, G. V., et al. (2013). Enrichr: Interactive and collaborative HTML5 gene list enrichment analysis tool. *BMC Bioinformatics* 14:128. doi: 10.1186/1471-2105-14-128

Chen, F., Wu, P., Deng, S., Zhang, H., Hou, Y., Hu, Z., et al. (2020). Dissimilation of synonymous codon usage bias in virus–host coevolution due to translational selection. *Nat. Ecol. Evol.* 4, 589–600. doi: 10.1038/s41559-020-1124-7

Coleman, J. R., Papamichail, D., Skiena, S., Futcher, B., Wimmer, E., and Mueller, S. (2008). Virus attenuation by genome-scale changes in codon pair bias. *Science* 320, 1784–1787. doi: 10.1126/science.1155761

Dana, A., and Tuller, T. (2014). The effect of tRNA levels on decoding times of mRNA codons. *Nucleic Acids Res.* 42, 9171–9181. doi: 10.1093/nar/gku646

Dhindsa, R. S., Copeland, B. R., Mustoe, A. M., and Goldstein, D. B. (2020). Natural selection shapes codon usage in the human genome. *Am. J. Hum. Genet.* 107, 83–95. doi: 10.1016/j.ajhg.2020.05.011

Diambra, L. A. (2017). Differential bicodon usage in lowly and highly abundant proteins. *PeerJ* 5:e3081. doi: 10.7717/peerj.3081

Feige, J.-J., Negoescu, A., Keramidas, M., Souchelnitskiy, S., and Chambaz, E. M. (1996). α2-macroglobulin: a binding protein for transforming growth factor-β and various cytokines. *Horm. Res. Paediatr.* 45, 227–232. doi: 10.1159/000184793

Frumkin, I., Lajoie, M. J., Gregg, C. J., Hornung, G., Church, G. M., and Pilpel, Y. (2018). Codon usage of highly expressed genes affects proteome-wide translation efficiency. *Proc. Natl. Acad. Sci. U.S.A.* 115, E4940–E4949. doi: 10.1073/pnas.1719375115

Gingold, H., and Pilpel, Y. (2011). Determinants of translation efficiency and accuracy. *Mol. Syst. Biol.* 7:481. doi: 10.1038/msb.2011.14

Girard, J. P., and Springer, T. A. (1995). Cloning from purified high endothelial venule cells of hevin, a close relative of the antiadhesive extracellular matrix protein SPARC. *Immunity* 2, 113–123. doi: 10.1016/1074-7613(95)90083-7

Girard, J. P., and Springer, T. A. (1996). Modulation of endothelial cell adhesion by hevin, an acidic protein associated with high endothelial venules. *J. Biol. Chem.* 271, 4511–4517. doi: 10.1074/jbc.271.8.4511

Gu, W., Zhou, T., Ma, J., Sun, X., and Lu, Z. (2004). Analysis of synonymous codon usage in SARS Coronavirus and other viruses in the Nidovirales. *Virus Res.* 101, 155–161. doi: 10.1016/j.virusres.2004.01.006

Haase, M., and Fitze, G. (2015). HSP90AB1: helping the good and the bad. *Gene* 575, 171–186. doi: 10.1016/j.gene.2015.08.063

Hamming, I., Timens, W., Bulthuis, M., Lely, A., Navis, G., and van Goor, H. (2004). Tissue distribution of ACE2 protein, the functional receptor for SARS coronavirus. A first step in understanding SARS pathogenesis. *J. Pathol.* 203, 631–637. doi: 10.1002/path.1570

Huang, C., Wang, Y., Li, X., Ren, L., Zhao, J., Hu, Y., et al. (2020). Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet* 395, 497–506. doi: 10.1016/S0140-6736(20)30183-5

Hulpke, S., and Tampé, R. (2013). The MHC I loading complex: a multitasking machinery in adaptive immunity. *Trends Biochem. Sci.* 38, 412–420. doi: 10.1016/j.tibs.2013.06.003

Hunt, R. C., Simhadri, V. L., Iandoli, M., Sauna, Z. E., and Kimchi-Sarfaty, C. (2014). Exposing synonymous mutations. *Trends Genet.* 30, 308–321. doi: 10.1016/j.tig.2014.04.006

Hussain, T., Tan, B., Ren, W., Rahu, N., Dad, R., Kalhoro, D. H., et al. (2017). Polyamines: therapeutic perspectives in oxidative stress and inflammatory diseases. *Amino Acids* 49, 1457–1468. doi: 10.1007/s00726-017-2447-9

Kandeel, M., Ibrahim, A., Fayez, M., and Al–Nazawi, M. (2020). From SARS and MERS CoVs to SARS–CoV–2: Moving toward more biased codon usage in viral structural and nonstructural genes. *J. Med. Virol.* 92, 660–666. doi: 10.1002/jmv.25754

Kenney, S. P., and Meng, X. J. (2015). An SH3 binding motif within the nucleocapsid protein of porcine reproductive and respiratory syndrome virus interacts with the host cellular signaling proteins STAMI, TXK, Fyn, Hck, and cortactin. *Virus Res.* 204, 31–39. doi: 10.1016/j.virusres.2015.04.004

Khosroshahi, H., Sahin, S., Akyuz, Y., and Ede, H. (2014). Long term follow-up of four patients with Keutel syndrome. *Am. J. Med. Genet. A* 164, 2849–2856. doi: 10.1002/ajmg.a.36699

Kim, A., Le Douce, J., Diab, F., Ferovova, M., Dubourg, C., Odent, S., et al. (2020). Synonymous variants in holoprosencephaly alter codon usage and impact the Sonic Hedgehog protein. *Brain* 143, 2027–2038. doi: 10.1093/brain/awaa152

Kirchner, S., Cai, Z., Rauscher, R., Kastelic, N., Anding, M., Czech, A., et al. (2017). Alteration of protein function by a silent polymorphism linked to tRNA abundance. *PLoS Biol.* 15:e2000779. doi: 10.1371/journal.pbio.2000779

Kwan, T., and Thompson, S. R. (2019). Noncanonical translation initiation in Eukaryotes. *Cold Spring Harbor Perspect. Biol.* 11:a032672. doi: 10.1101/cshperspect.a032672

Le Nouen, C., Brock, L. G., Luongo, C., McCarty, T., Yang, L., Mehedi, M., et al. (2014). Attenuation of human respiratory syncytial virus by genome-scale codon-pair deoptimization. *Proc. Natl. Acad. Sci. U.S.A.* 111, 13169–13174. doi: 10.1073/pnas.1411290111

Lee, K., Kang, H.-S., Jeon, J., Kim, E.-M., Yoon, S.-R., Song, H., et al. (2005). VDUP1 is required for the development of natural killer cells. *Immunity* 22, 195–208. doi: 10.1016/j.immuni.2004.12.012

Maruyama, T., Nara, K., Yoshikawa, H., and Suzuki, N. (2007). Txk, a member of the non-receptor tyrosine kinase of the Tec family, forms a complex with poly(ADP-ribose) polymerase 1 and elongation factor 1α and regulates interferon-γ gene transcription in Th1 cells. *Clin. Exp. Immunol.* 147, 164–175. doi: 10.1111/j.1365-2249.2006.03249.x

McCarthy, C., Carrea, A., and Diambra, L. (2017). Bicodon bias can determine the role of synonymous SNPs in human diseases. *BMC Genomics* 18:227. doi: 10.1186/s12864-017-3609-6

Munroe, P. B., Olgunturk, R. O., Fryns, J. P., Van Maldergem, L., Ziereisen, F., Yuksel, B., et al. (1999). Mutations in the gene encoding the human matrix Gla protein cause Keutel syndrome. *Nat. Genet.* 21, 142–144. doi: 10.1038/5102

Nicholson, P., Yepiskoposyan, H., Metze, S., Orozco, R. Z., Kleinschmidt, N., Mühlemann, O., et al. (2010). Nonsense-mediated mRNA decay in human cells: Mechanistic insights, functions beyond quality control and the double-life of NMD factors. *Cell. Mol. Life Sci.* 67, 677–700. doi: 10.1007/s00018-009-0177-1

Nikolich-Zugich, J., Knox, K. S., Rios, C. T., Natt, B., Bhattacharya, D., and Fain, M. J. (2020). SARS-CoV-2 and COVID-19 in older adults: what we may expect regarding pathogenesis, immune responses, and outcomes. *GeroScience* 42, 505–514. doi: 10.1007/s11357-020-00186-0

Pan, A., Dutta, C., and Das, J. (1998). Codon usage in highly expressed genes of *Haemophillus influenzae* and *Mycobacterium tuberculosis*: translational selection versus mutational bias. *Gene* 215, 405–413. doi: 10.1016/S0378-1119(98)00257-1

Pankov, R., and Yamada, K. M. (2002). Fibronectin at a glance. *J. Cell Sci.* 115, 3861–3863. doi: 10.1242/jcs.00059

Pérez-Cano, F. J., Franch, À., Castellote, C., and Castell, M. (2003). Immunomodulatory action of spermine and spermidine on NR8383 macrophage line in various culture conditions. *Cell. Immunol.* 226, 86–94. doi: 10.1016/j.cellimm.2003.09.009

Piovesan, A., Vitale, L., Pelleri, M. C., and Strippoli, P. (2013). Universal tight correlation of codon bias and pool of RNA codons (codonome): the genome is optimized to allow any distribution of gene expression values in the transcriptome from bacteria to humans. *Genomics* 101, 282–289. doi: 10.1016/j.ygeno.2013.02.009

Plotkin, J. B., and Kudla, G. (2011). Synonymous but not the same: the causes and consequences of codon bias. *Nat. Rev. Genet.* 12, 32–42. doi: 10.1038/nrg2899

Quax, T. E., Claassens, N. J., Söll, D., and van der Oost, J. (2015). Codon bias as a means to fine-tune gene expression. *Mol. Cell* 59, 149–161. doi: 10.1016/j.molcel.2015.05.035

Schroder, K., Hertzog, P. J., Ravasi, T., and Hume, D. A. (2004). Interferon-γ: an overview of signals, mechanisms and functions. *J. Leukoc. Biol.* 75, 163–189. doi: 10.1189/jlb.0603252

Shackelton, L. A., Parrish, C. R., and Holmes, E. C. (2006). Evolutionary basis of codon usage and nucleotide composition bias in vertebrate DNA viruses. *J. Mol. Evol.* 62, 551–563. doi: 10.1007/s00239-005-0221-1

Shah, P., and Gilchrist, M. A. (2011). Explaining complex codon usage patterns with selection for translational efficiency, mutation bias, and genetic drift. *Proc. Natl. Acad. Sci. U.S.A.* 108, 10231–10236. doi: 10.1073/pnas.1016719108

Shen, B., Yi, X., Sun, Y., Bi, X., Du, J., Zhang, C., et al. (2020). Proteomic and metabolomic characterization of COVID-19 patient sera. *Cell* 182, 59–72. doi: 10.1016/j.cell.2020.05.032

Surjit, M., Liu, B., Jameel, S., Chow, V. T., and Lal, S. K. (2004). The SARS coronavirus nucleocapsid protein induces actin reorganization and apoptosis in COS-1 cells in the absence of growth factors. *Biochem. J.* 383, 13–18. doi: 10.1042/BJ20040984

Thoms, M., Buschauer, R., Ameismeier, M., Koepke, L., Denk, T., Hirschenberger, M., et al. (2020). Structural basis for translational shutdown and immune evasion by the Nsp1 protein of SARS-CoV-2. *Science* 17:eabc8665. doi: 10.1126/science.abc8665

Tsai, C.-J., Sauna, Z. E., Kimchi-Sarfaty, C., Ambudkar, S. V., Gottesman, M. M., and Nussinov, R. (2008). Synonymous mutations and ribosome stalling can lead to altered folding pathways and distinct minima. *J. Mol. Biol.* 383, 281–291. doi: 10.1016/j.jmb.2008.08.012

Tuller, T., Carmi, A., Vestsigian, K., Navon, S., Dorfan, Y., Zaborske, J., et al. (2010a). An evolutionarily conserved mechanism for controlling the efficiency of protein translation. *Cell* 141, 344–354. doi: 10.1016/j.cell.2010.03.031

Tuller, T., Waldman, Y. Y., Kupiec, M., and Ruppin, E. (2010b). Translation efficiency is determined by both codon bias and folding energy. *Proc. Natl. Acad. Sci. U.S.A.* 107, 3645–3650. doi: 10.1073/pnas.0909910107

von Herrmann, K. M., Salas, L. A., Martinez, E. M., Young, A. L., Howard, J. M., Feldman, M. S., et al. (2018). NLRP3 expression in mesencephalic neurons and characterization of a rare NLRP3 polymorphism associated with decreased risk of Parkinson's disease. *NPJ Parkinsons Dis.* 4:24. doi: 10.1038/s41531-018-0061-5

Wang, Y., and Ni, H. (2016). Fibronectin maintains the balance between hemostasis and thrombosis. *Cell. Mol. Life Sci.* 73, 3265–3277. doi: 10.1007/s00018-016-2225-y

White, M. P., Theodoris, C. V., Liu, L., Collins, W. J., Blue, K. W., Lee, J. H., et al. (2015). NOTCH1 regulates matrix gla protein and calcification gene networks in human valve endothelium. *J. Mol. Cell. Cardiol.* 84, 13–23. doi: 10.1016/j.yjmcc.2015.04.006

Xia, C., Vijayan, M., Pritzl, C. J., Fuchs, S. Y., McDermott, A. B., and Hahm, B. (2016). Hemagglutinin of influenza a virus antagonizes type I interferon (IFN) responses by inducing degradation of Type I IFN receptor 1. *J. Virol.* 90, 2403–2417. doi: 10.1128/JVI.02749-15

Zhou, T., Weems, M., and Wilke, C. O. (2009). Translationally optimal codons associate with structurally sensitive sites in proteins. *Mol. Biol. Evol.* 26, 1571–1580. doi: 10.1093/molbev/msp070

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Public Health

SYSTEMATIC REVIEW
published: 20 August 2020
doi: 10.3389/fpubh.2020.00487

An Overview of the Temporal Shedding of SARS-CoV-2 RNA in Clinical Specimens

Khrystyna Zhurakivska, Giuseppe Troiano, Giuseppe Pannone, Vito Carlo Alberto Caponio and Lorenzo Lo Muzio*

Department of Clinical and Experimental Medicine, University of Foggia, Foggia, Italy

Coronavirus disease 2019 quickly spread in China and has, since March 2020 become a pandemic, causing hundreds of thousands of deaths worldwide. The causative agent was promptly isolated and named SARS-CoV-2. Scientific efforts are related to identifying the best clinical management of these patients, but also in understanding their infectivity in order to limit the spread of the virus. Aimed at identifying viral RNA in the various compartments of the organism of sick subjects, diagnostic tests are carried out. However, the accuracy of such tests varies depending on the type of specimen used and the time of illness at which they are performed. This review of the literature aims to summarize the preliminary findings reported in studies on Covid-19 testing. The results highlight how the pharyngeal swab is highly sensitive in the first phase of the disease, while in the advanced stages, other specimens should be considered, such as sputum, or even stool to detect SARS-CoV-2. It highlights that most patients already reach the peak of the viral load in the upper airways within the first days of displaying symptoms, which thereafter tend to decrease. This suggests that many patients may already be infectious before symptoms start to appear.

Keywords: coronavirus 2019, Covid-19, SARS-CoV-2, specimens, pharyngeal swabs, feces, sputum

OPEN ACCESS

Edited by:
Longxiang Su,
Peking Union Medical College Hospital (CAMS), China

Reviewed by:
Tarek A. Ahmad,
Bibliotheca Alexandrina, Egypt
Juanjuan Zhao,
Shenzhen Third People's Hospital, China
Jing Yuan,
Children's Hospital of Capital Institute of Pediatrics, China

****Correspondence:***
Giuseppe Troiano
giuseppe.troiano@unifg.it;
giutroiano@gmail.com

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Public Health

Received: *07 May 2020*
Accepted: *30 July 2020*
Published: *20 August 2020*

Citation:
Zhurakivska K, Troiano G, Pannone G, Caponio VCA and Lo Muzio L (2020) An Overview of the Temporal Shedding of SARS-CoV-2 RNA in Clinical Specimens. Front. Public Health 8:487. doi: 10.3389/fpubh.2020.00487

INTRODUCTION

Coronavirus disease 2019 (COVID-19) quickly spread in China was declared a became pandemic in March 2020, causing hundreds of thousands of deaths worldwide. The causative agent, a virus from the coronavirus family, was promptly isolated and named SARS-CoV-2 (1). The characteristics that make this virus highly dangerous for the population are represented by a very high transmission capacity, as well as by its complex interaction with the host's organism which in a variable, but high percentage of cases, can lead to death (2). Transmission through respiratory droplets, indirect contact, as well as airborne transmission of the virus has been confirmed and the diagnosis is made combining clinical, radiographic (chest Computer Tomography), and laboratory evaluations. In particular, the presence of viral RNA in the pharyngeal swab is analyzed using the real-time reverse transcription-polymerase chain reaction (RT-PCR) (3–5). Regarding the molecular targets that can be used for PCR assays, some structural proteins were identified, among which: spike (S), envelope (E), transmembrane (M), helicase (Hel), and nucleocapsid (N). Furthermore, other genes that are required for viral replication, like RNA-dependent RNA polymerase (RdRp),

hemagglutinin-esterase (HE), and open reading frames (ORF1ab) may be targeted for virus detection by RT-PCR (4, 6). There are different recommendations among countries regarding the choice of target (4, 7), nevertheless, to obtain a reliable result, at least two molecular targets should be included in the assay (8). The result of RT-PCR, expressed in Cycle threshold (Ct) provides an answer about the presence or absence of the viral RNA and also estimates the viral load in the sample, where the Ct is inversely proportional to the quantity of the viral RNA. Even if so, it seems that positivity diagnosed with RT-PCR is not indicative of the contagiousness of the patient (1).

Scientific efforts at this time are directed on multiple fronts: on the one hand, researchers are studying the best clinical management of infected patients; on the other hand, they are trying to define the infectious aspects of these patients. In particular, it is necessary to understand when the SARS-CoV-2 positive subject is capable of infecting others or when this possibility is greater? In which biological materials is the virus present and in what quantities? How do these values change during the course of the disease? Are they related to the symptoms?

Partial answers to these questions come from an increasing number of studies that have reported the clinical and virological data of patients, observed in various parts of the world. However, these data often relate to a few patients or only focus on some aspects and not others.

This review aims to summarize the findings of the studies published until now regarding the trend of temporal shedding of SARS-CoV-2 RNA in various clinical specimens.

MATERIALS AND METHODS

The electronic database PubMed was screened in order to select studies suitable for inclusion in this review. The following strategy of search was used: [("SARS-CoV-2" OR "2019-nCoV" OR "covid-19") AND (load OR samples OR specimens)]. In addition, bibliographies of the included studies were read, and suitable references researched separately.

The results were screened by title and abstract, selecting the records fulfilling the following inclusion criteria:

- studies published in English;
- studies reporting data on SARS-CoV2 RNA evaluation in clinical specimens with chronological reference to the illness course.

No restrictions on the study design were applied.

The established exclusion criteria consisted of:

- studies written in languages other than English;
- studies evaluating treatment options;
- non-original studies;
- studies without a clear reference to the onset of the disease (onset of symptoms).

In case of insufficient information after abstract reading, the full-text publication was examined.

The selected papers were full-text evaluated and, if meeting the inclusion criteria, were included in the review.

An *ad hoc* datasheet containing queries was prepared and the following data, if available, was extracted and inserted into the datasheet:

- Author's names;
- number of patients;
- type of specimen analyzed and results of RT-PCR with the corresponding days of illness from symptom onset to which they refer;
- molecular target used in the RT-PCR analysis.

Qualitative Analysis

The results of the examined specimens reported for every day of patients' illness were collected. If the result of the test was positive, according to the parameters established in the original paper, a "+" was assigned, while a "–" was assigned if the test result was negative.

No distinction was made on the methodology used in the various studies, nor on the unit of measure, only a dichotomous result (+ or –) was reported.

The total percentage of positives and negatives was thus determined day by day, for each type of sample.

Quantitative Analysis

The cases for which the Ct values of RT-PCR were reported for every single test were included in this analysis. The data were grouped by type of target (i.e., ORF1ab, E, S, RdRp etc.) used for virus RNA detection in every type of specimen. The mean and standard deviation of Ct values were calculated for each day of patients' illness.

Other Analysis

The descriptive results that could not be included neither in a quantitative nor in a qualitative analysis, were also collected.

RESULTS

A total of 243 records were found, applying the search strategy on electronic databases. After the title and abstract examination, 25 abstracts fulfilled the inclusion criteria and were selected for a full-text reading. Of these, 21 (7, 9–27) were deemed suitable for inclusion in the review. Generic information about the included studies are reported in **Table 1**.

The discarded articles were focused on the evaluation of some treatments and therefore considered misleading for the purposes of our evaluation (29–31). One study was only a descriptive report and was also excluded (32). A flowchart representing the selection process is reported in **Figure 1**.

Due to a large variability among studies in methodology and presentation of results, only six studies were included in the quantitative evaluation, while for others a qualitative or discursive consideration was performed.

Qualitative Results

In the final qualitative analysis 68 patients were included. Of these, complete temporal data, with reference to the

TABLE 1 | Main characteristics of included studies.

Author	N° of patients	Country	Investigated specimens	N° patients included in quantitative analysis	N° patients included in qualitative analysis
Chen et al. (10)	57	China	Pharyngeal swab, blood, anal swab,	6	6
Chen et al. (11)	42	China	pharyngeal swab, stool, urine	0	0
Holshue et al. (7)	1	USA	Naso-and oropharyngeal swabs, blood, feces, urine	0	1
Kam et al. (13)	2	Singapore	Pharyngeal swab, blood, feces, urine, mother's breast milk	1	1
Kim et al. (12)	2	Korea	Naso- and oropharyngeal swabs, serum, plasma, sputum, feces, urine	2	2
Lan et al. (9)	4	China	Oropharyngeal swabs	0	0
Lescure et al. (14)	5	France	Pharyngeal swab, plasma, feces, urine, conjunctiva	0	5
Liu et al. (27)	12	China	Oropharyngeal swab, Bronchoalveolar lavage, Fluid	6	6
Lo et al. (15)	10	China	Nasopharyngeal swab, sputum, urine, feces	0	0
Pan et al. (16)	82	China	Oropharyngeal swab, sputum, feces, urine	0	2
Qiu et al. (28)	10	China	Vaginal fluids	0	0
To et al. (17)	12	China	Saliva	0	0
To et al. (18)	23	China	Blood, saliva, anal swab, urine	0	0
Wang et al. (19)	205	China	Pharyngeal swab, blood, sputum, nasal swab, bronchoalveolar lavage fluid, Fibrobronchoscope brush biopsy, feces, urine	0	0
Wölfel et al. (23)	9	Germany	Pharyngeal swab, sputum, feces	0	9
Xiao et al. (24)	73	China	Pharyngeal swab, stool	0	0
Yang et al. (26)	213	China	nasal swabs, throat swabs, sputum, bronchoalveolar lavage fluid	0	0
Young et al. (20)	18	Singapore	Nasopharyngeal swab, blood, feces, urine	18	18
Yu et al. (21)	76	China	Nasopharyngeal swab, oropharyngeal swabs, plasma, sputum, nasal swab, urine	0	0
Zhang et al. (22)	15	China	Oral swab, anal swab, blood	0	0
Zou et al. (25)	18	China	Oropharyngeal swab, nasopharyngeal swab	18	18

day of illness were available for: pharyngeal specimens in 68 patients; blood specimens in 28 patients; feces samples in 25 patients; urine in 17 patients; sputum in 13 patients. The main findings of data analysis revealed that:

- most patients had a positive Pharyngeal swab result for the first 10 days of illness. After this term, the percentage of patients whose Pharyngeal swab result was negative increased, and then even exceeded the positive ones around day 12 of illness (**Figure 2**);
- viral RNA was not detected in the blood of most patients. In <15% of patients, viremia was registered in the second week of illness (**Figure 3**);
- sputum contains viral RNA throughout the duration of the disease (**Figure 4**);
- the virus is eliminated in the stool of sick patients. Toward the end of the first week of the disease, viral RNA was found in approximately 40% of patients (**Figure 5**);
- the urine of Covid-19 patients was almost always negative for the presence of the virus (**Figure 6**).

The **Figure 7** summarizes the percentages of positivity observed for each type of specimen during the patients' illness.

Quantitative Results

Data related to 51 patients were included in the quantitative analysis. The table included in the **Supplementary Material** summarizes information about the analyzed specimens, molecular targets used, and the Ct values observed at RT-PCR analysis.

The time course of RT-PCR Ct values related to the most representative specimens are reported in **Figure 8** in a cumulative way, regardless of the type of molecular target. The representation of Ct values in specimens, divided by type of molecular target are present in the **Supplementary Materials**.

Other results reported in the included studies which were not considered in the quantitative and qualitative analyses, affirm as follows:

Upper Respiratory Samples

Pharyngeal viral load is highest in the early phase of illness (12, 16, 20–23, 25), showing high levels already in the first 24 h from the onset of symptoms (14) with the peak on the 5–6th day of illness (16). According to Wolfel et al., in this period the detection rate was 100%, decreasing substantially after day 5, with a detection rate that more than halves (39.93%) (23). Furthermore, the study of subgenomic messenger RNAs (sgRNA) suggested

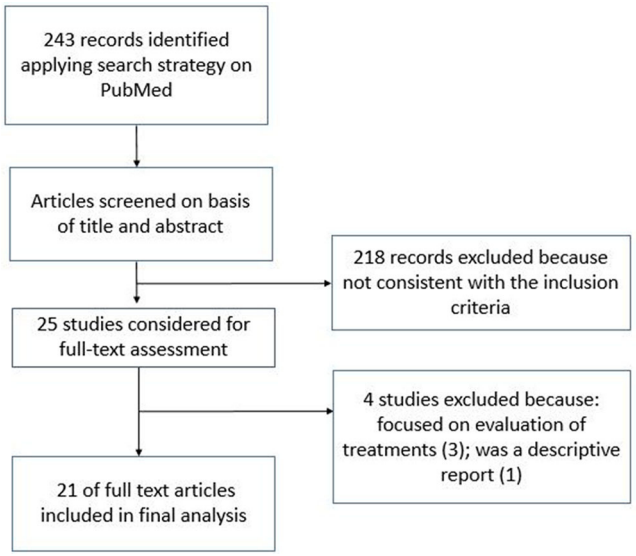


FIGURE 1 | Flowchart representing the selection process of the studies suitable for inclusion.

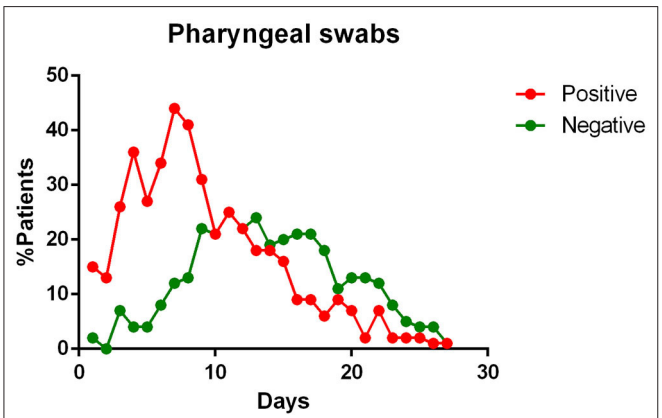


FIGURE 2 | Positivity and negativity rates of Pharyngeal specimens along the time of illness. Each dot represents the percentage of the analyzed specimens that resulted positive or negative on that specific day.

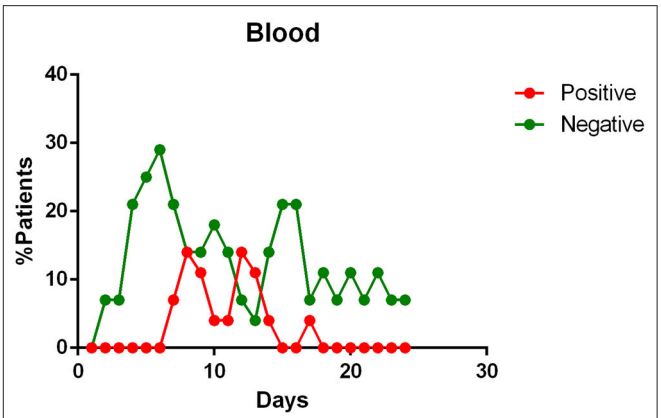


FIGURE 3 | Positivity and negativity rates of Blood specimens along the time of illness. Each dot represents the percentage of the analyzed specimens that resulted positive or negative on that specific day.

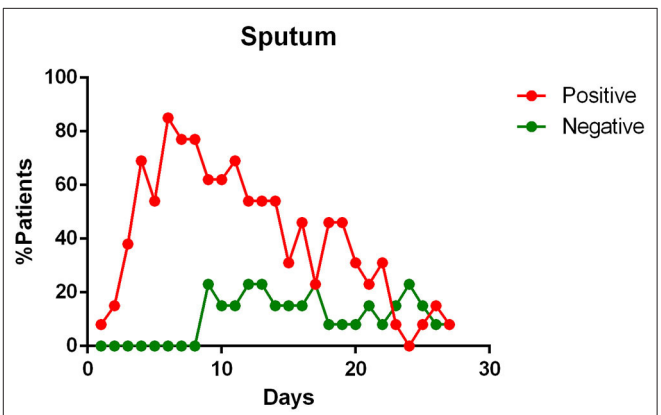


FIGURE 4 | Positivity and negativity rates of Sputum along the time of illness. Each dot represents the percentage of the analyzed specimens that resulted positive or negative on that specific day.

that the first 5 days of illness are characterized by an active replication of SARS-CoV-2 in the upper respiratory tract, while after the 5th day, no sgRNA was detected in pharyngeal samples (23). In the advanced stage of the disease (second–third week), the virus can be intermittently detectable in nasopharyngeal swabs (9, 20).

Some authors reported a positive correlation between the severity of clinical conditions and upper respiratory tract viral load (25, 27).

Regarding the comparison of naso-and oropharyngeal swabs, the opinions are discordant: Wölfel et al. (23) state that no differences in viral loads or detection rates were revealed when comparing naso- and oropharyngeal swabs, while Zou et al. (25) and Yang et al. (26) noticed higher viral loads and detection rates in the nose swabs. Yu et al. (21), contrariwise, found a higher mean viral load in the throat (2,552 vs. 651 copies/test, $p < 0.001$).

Blood Specimens

Blood positivity rates reported among COVID-19 patients vary between 0 and 22% (10, 12, 14, 18–21, 23). Chen et al. affirm that the detection of viral RNA in the blood is a strong indicator of illness severity (10).

Feces Specimens and Anal Swabs

Stool content of viral RNA was detected in a great percentage of patients enrolled in various studies (11, 14, 19, 20, 23, 24). Wolfel et al. noted that the viral load in the stool seemed to reflect the sputum viral content (23).

Several authors therefore suppose an infection of the gastro-intestinal tract by the virus (11, 24), with its continuous elimination with the feces which has been reported to last from 1 to 12 days (24) and in some cases, viral RNA were detected in feces or anal swabs even after the respiratory tests became

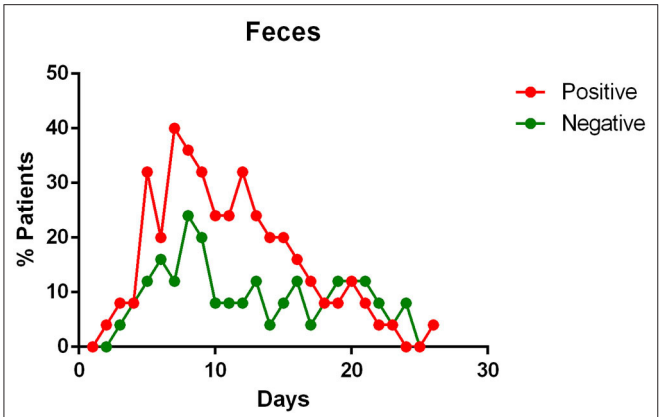


FIGURE 5 | Positivity and negativity rates of Feces along the time of illness. Each dot represents the percentage of the analyzed specimens that resulted positive or negative on that specific day.

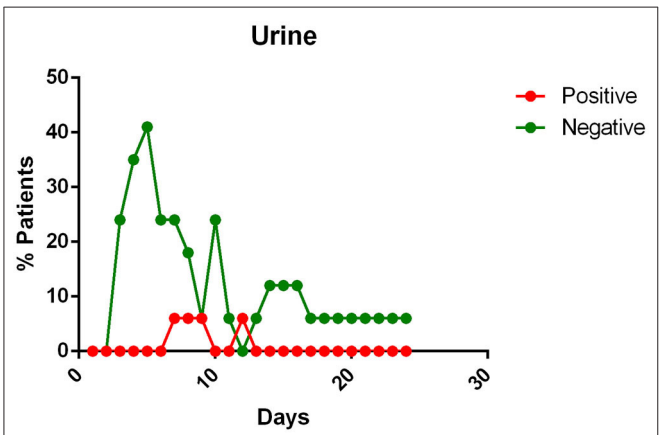


FIGURE 6 | Positivity and negativity rates of Urine along the time if illness. Each dot represents the percentage of the analyzed specimens that resulted positive or negative on that specific day.

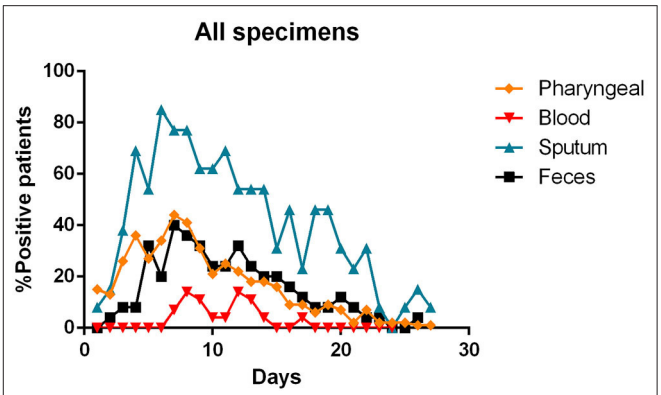


FIGURE 7 | Percentages of positivity observed for the main types of specimens during the illness of included patients. Each dot represents the percentage of the analyzed specimens that resulted positive or negative on that specific day.

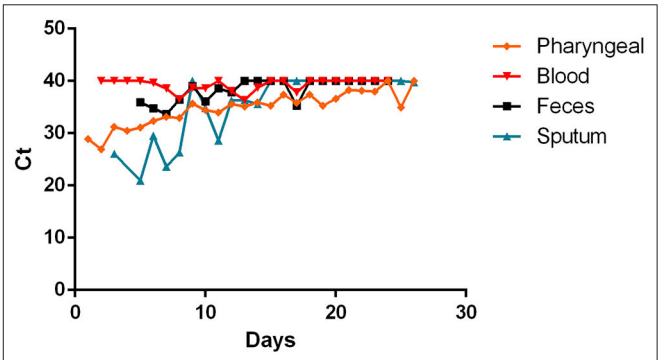


FIGURE 8 | The time course of RT-PCR Ct values in main specimens.

negative (11, 22, 24). Zhang et al. also report that during the first days of illness, the most positive swabs were the oral ones, whereas in the following days more and more anal swabs were positive, and oral ones negative (22).

Sputum Samples

Sputum samples appear to contain the maximum viral load (16, 21, 23), reaching the peak on the 5–6th day after symptoms onset (16) and remain positive for a maximum duration over time, compared to swabs of the upper respiratory tract (23). They also show one of the highest positivity rates (53.42–100%) among the tested samples (19, 21, 23, 24, 26), giving positive results for a long time, even when the pharyngeal samples are negative for the presence of the SARS-CoV-2 RNA (15), sometimes even after symptoms have ended (23). Some authors state that the sputum viral load seems to be significantly correlated to the pharyngeal one (12, 16).

Urine

All patients, except one in Kim's report (12) and four reported by Liu et al. (27) had negative viral detection in urine.

Saliva

Results on viral RNA detection in saliva are reported in two papers (17, 18). The detection rate in the initial samples is estimated to be around 90% (17, 18). The serial daily sampling revealed that the viral load was highest during the first week of symptoms and declined in the following days. On day 20 after symptoms onset, 33% of patients had viral RNA detected in the saliva specimens (18).

The main findings of the included studies are summarized in the **Table 2**.

DISCUSSION

The pandemic spread of coronavirus infection SARS-CoV-2 forced many countries to take strong containment measures (33, 34). To avoid an uncontrolled broadcast of the disease, it is fundamental to understand the manner and timing of disease transmission. Then, a reliable test is needed to identify infected subjects, to take appropriate isolation measures for a period sufficient enough to avoid contagion of other individuals.

TABLE 2 | Main findings of the included studies.

	Main findings in specimens							Notes
Author	**Pharyngeal swabs**	**Blood/plasma/serum**	**Sputum**	**Feces**	**Urine**	**Anal swabs**	**Saliva**	
Chen et al. (10)		Positive in 6/57 (10,52%) of patients				Positive in 11/28 (39,28%) patients		Positive correlation of serum viral RNA with the disease severity supposed.
Chen et al. (11)				Positive in 28/40 (66.67%) patients	Positive in 0/10 (0%) patients			18/28 (64.29%) patients remained positive for viral RNA in feces for 7 (6–10) days after pharyngeal swabs turned negative
Lan et al. (9)								All patients, had 2 consecutive negative RT-PCR tests during recovering stage, returning to be positive 10–18 days later
Lescure et al. (14)	Maximum viral load in the first days of illness	Positive in 1/5 (20%) patients		Positive in 2/5 (40%) patients	All negative			
Liu et al. (27)					Positive in 4/6 (66,66%) patients			The viral load detected from respiratory tracts was positively linked to lung disease severity
Lo et al. (15)	9/10 (90%) positive at the first test			10/10 (100%) positive at the first test	Positive in 0/10 (0%) patients			
Kam et al. (13)	Positive in 2/2 (100%) patients	Positive only 1 day in 1 patient		1 positive value during illness course				
Kim et al. (12)	Positive in 2/2 (100%) patients	Few positive values during the illness course	Positive in 2/2 (100%)		Positive in 1/2 (50%) patient			
Pan et al. (16)	High viral load early after onset		High viral load early after onset					Viral loads of pharyngeal and sputum samples were significantly correlated
To et al. (17)							First specimens: positive in 91.66% of patients	
To et al. (18)		First specimen: positive in 22% of patients			All negative	First specimen positive in 27% of patients	First specimen: positive in 87%	
Wang et al. (19)	Positive in 126/398 (32%) of samples	Positive in 3/307 (1%) of samples	Positive in 75/104 (72%) of samples	Positive in 44/153 (29%) of samples	Positive 0/72 (0%) of samples			
Wölfel et al. (23)	Positive in 100% of cases on days 1–5	All negative	Positive in 100% of patients	Positive in 89% of patients	Positive in 0/9 (0%) patients			
Xiao et al. (24)			Positive 39/73 (53.42%) patients					17/39 (43.58%) patients remained positive in stool after showing negative in respiratory samples

(Continued)

TABLE 2 | Continued

Author	Main findings in specimens							Notes
	Pharyngeal swabs	Blood/plasma/serum	Sputum	Feces	Urine	Anal swabs	Saliva	
Yang et al. (26)	Nasal/oral swabs positive in 73%/60% of cases in early stage.		Positive in 85% of samples in early stage					
Young et al. (20)	Positive in 100% Positive over 7 days in 83%	Positive in 1/12 (8.33%) patients		Positive in 4/8 (50%) patients	All negative			
Yu et al. (21)	Positive in 9/55 (16.4%) of nasal swabs, 50/134 (37.3%) of throat swabs	Positive in 0/4 (0%) samples	Positive in 77/116 (66.4%) samples		Positive in 0/14 (0%) samples			Analyzed with ddPCR
Zhang et al. (22)	More positive in early period					More positive in later period		
Zou et al. (25)	Viral load higher in patients with severe illness condition							

The reference method for testing positivity to SARS-CoV-2 infection is represented by the pharyngeal swab that is taken from the patient's nasopharynx or oropharynx and, through an RT-PCR analyzed for the presence of viral RNA (8). This method has been reasonably chosen, as it has already been used for other viruses affecting the airway tract, such as SARS-CoV (35). The wide use of such protocol is due to its multiple advantages. It is simple to perform, relatively inexpensive, and fast. However, as has emerged from recent studies, and confirmed by our cumulative analysis, the accuracy in the diagnosis of this swab seems to be excellent in the first phase of the disease, losing sensitivity in the following days (16, 20–23, 25). This can be linked to a reduction in the viral load present in the upper respiratory tracts starting from the second week of the disease (14, 16, 20, 23).

These data reveal two aspects to reflect on. The first one concerns the initial phase of the disease, that is, when the symptoms arise and the viral load in the upper airways is already almost at the peak, as suggested by several authors (14, 16, 23). This implies that many patients may be infectious for days before they show signs of disease. The second reflection concerns the terminal phase of the disease. In particular, attention should be paid to patients who test negative for the pharyngeal swab in the advanced stages of the disease, since Young et al. (20) and Lan et al. (9) show that the swab may be positive intermittently in this phase. Therefore, it is fundamental to understand whether the virus can be transmitted in this stage of disease. The presentation of the results of the RT-PCR analysis, however, remains only for a diagnostic purpose, without being able to provide indications on the contagiousness of the positive subject. Other methods like isolation and culture of the virus, are needed for this estimate (8).

For diagnostic proposes, it should be considered, as stated by Yu et al., that the performance of a droplet digital PCR (ddPCR) in SARS-CoV-2 detection may be significantly better compared to the traditional RT-PCR, especially for low viral loads (21).

In addition, according to some authors, there seems to be a difference between nasopharyngeal and oropharyngeal swabs (21, 25). In particular, one study with a high overall number of performed swabs (250 throat and 490 nasal swabs) state that the nasal swabs have a significantly higher positive rate than the oropharyngeal ones (73.3% vs. 60% in the first 7 days and 72.3% vs. 50.0% during the second week of illness) (26).

Among the investigated samples, saliva seems to be a promising specimen for detection of SARS-CoV-2 (17, 18). Authors found a positivity rate in the initial saliva samples of 87%, with a median viral load of 3.3×10^6 copies per mL, values that seem to be similar to the pharyngeal swabs (ranging between 104 and 107 copies per mL) (16). The temporal course of the viral load in the saliva seems to follow that of the pharynx (18), even if it was not possible to refer data to the symptoms onset, but only to the hospitalization timing.

The sputum, seems to possess the highest positive rate among all the specimens (26), except for bronchoalveolar lavage fluid (BALF) (19, 26), and persists throughout the course of the disease (21, 23, 24, 26). The study investigating the active viral replication in the cells using sgRNA, found that the active replication of SARS-CoV-2 in the sputum samples persisted until days 10/11

of the illness, unlike pharyngeal swabs, where sgRNAs were no longer detectable at the end of the first week of symptoms (23). As suggested by Lo et al. (15) the sputum could be useful in the diagnosis of some suspected cases that are negative with repeated pharyngeal swabs.

Regarding the advanced stages of the disease, a fair rate of SARS-CoV-2 positivity was found in the stool of infected patients. In studies investigating the presence of viral RNA in the feces, more than half [and up to 90% reported by Lo et al. (15)] of the patients tested positive (11, 24). Furthermore, sometimes the fecal specimen remained positive, even after the pharyngeal specimen became negative (11, 22, 24). We do not know what implications this data has on the transmission or on the course of the disease, however, fecal examination should be considered to complement the diagnosis of COVID-19 patients.

The presence of viral RNA in the blood has also been investigated. However, few patients appear to have viremia during the course of the disease (14, 18, 19). Although, this event appears to be positively correlated with the severity of the symptoms (10).

No viral RNA was detected in breast milk (13), nor in vaginal fluids (28) of infected women.

An attempt was made in this overview to compare the Ct values of the main specimens that were found in the various studies during the course of the disease. Surely this result may be affected by a bias due to the difference in the methods and targets used in the various studies, even if there are universally accepted cut-offs (Ct-value < 40) that give us a reference in the interpretation of the results (8).

Another important aspect regarding the SARS-CoV-2 genome, and thanks to the availability of the newest sequencing methods and highly organized databases, several researchers are investigating genetic characteristics of the virus, subtype evolution, as well as geographic and temporal changes in the virus genome. Major attention has been focused on homoplasies, that is mutations that have emerged multiple times and may represent the sign of ongoing adaptation of the virus to the new human host. Several mutations in different regions of the viral genome have been found. These include sites in the Orf1ab region, Spike protein (36, 37), as well as the N gene (38). The implications of such mutations are not completely known. Some of them can be neutral (39), but it can be supposed that the changes in surface glycoprotein can influence the interaction between the virus and the host cell, as well as the anti-genicity of the virus (36, 40, 41).

A great part of knowledge about the genomic stability of SARS-CoV2 is still in evolving. It is still unclear if some sequence differences found in samples coming from different continents represent a temporal rather than a geographic signal. Further studies are needed to better define the behavior of the virus, in order to develop efficient treatments.

CONCLUSIONS

A comprehensive approach of this overview was chosen in order to include as much data as possible in the final analysis, making it possible to analyze the data related to 889 patients, while all data reported the results differently. The results in the included studies were reported unevenly. Some were reports of a few patients, others presented data for many patients, but in a synthetic way. For this reason, the homogeneous data have been grouped together as far as possible and others treated discursively. However, some important conclusions emerged:

- the sputum, together with the bronchoalveolar lavage fluid, closely reflect the course of the infection;
- the pharyngeal swabs have a high accuracy in the initial phase of the disease, while their positivity rate drops suddenly in the following phases;
- viral RNA could be eliminated in the stool even for prolonged periods and their examination could supplement the pharyngeal swab.

Further studies with standardized protocols and an equally large number of samples for all types of specimens would be needed to draw more precise conclusions.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. The data can be found in papers cited in the References.

AUTHOR CONTRIBUTIONS

KZ and GP conceived the research. GT, KZ, and VC performed the literature search, analyzed the data, and wrote the article. LL supervised the research. All authors contributed to the article and approved the submitted version.

FUNDING

This study was funded by the authors' institution.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2020.00487/full#supplementary-material

REFERENCES

1. Wu F, Zhao S, Yu B, Chen YM, Wang W, Song ZG, et al. A new coronavirus associated with human respiratory disease in China. *Nature*. (2020) 579:265–9. doi: 10.1038/s41586-020-2008-3
2. Cheng VCC, Wong SC, Chen JHK, Yip CCY, Chuang VWM, Tsang OTY, et al. Escalating infection control response to the rapidly evolving epidemiology of the coronavirus disease 2019 (COVID-19) due to SARS-CoV-2 in Hong Kong. *Infect Control Hosp Epidemiol*. (2020) 1:1–6. doi: 10.1017/ice.2020.58
3. Chu DKW, Pan Y, Cheng SMS, Hui KPY, Krishnan P, Liu Y, et al. Molecular diagnosis of a novel coronavirus (2019-nCoV) causing an outbreak of pneumonia. *Clin Chem*. (2020) 66:549. doi: 10.1093/clinchem/hvaa029

4. Corman VM, Landt O, Kaiser M, Molenkamp R, Meijer A, Chu DK, et al. Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. *Euro Surveill*. (2020) 25:2000045. doi: 10.2807/1560-7917.ES.2020.25.3.2000045
5. Yang C, Ma QY, Zheng YH, Yang YX. [Transmission routes of 2019-novel coronavirus. (2019-nCoV)]. *Zhonghua Yu Fang Yi Xue Za Zhi*. (2020) 54:374–7. doi: 10.3760/cma.j.cn112150-20200216-0016
6. Lu R, Zhao X, Li J, Niu P, Yang B, Wu H, et al. Genomic characterisation and epidemiology of 2019 novel coronavirus: implications for virus origins and receptor binding. *Lancet*. (2020) 395:565. doi: 10.1016/S0140-6736(20)30251-8
7. Holshue ML, DeBolt C, Lindquist S, Lofy KH, Wiesman J, Bruce H, et al. First case of 2019 novel coronavirus in the United States. *N Engl J Med*. (2020) 382:929–36. doi: 10.1056/NEJMoa2001191
8. Tang YW, Schmitz JE, Persing DH, Stratton CW. The laboratory diagnosis of COVID-19 infection: current issues and challenges. *J Clin Microbiol*. (2020) 58:e00512-20. doi: 10.1128/JCM.00512-20
9. Lan L, Xu D, Ye G, Xia C, Wang S, Li Y, et al. Positive RT-PCR test results in patients recovered from COVID-19. *JAMA*. (2020) 323:1502–3. doi: 10.1001/jama.2020.2783
10. Chen W, Lan Y, Yuan X, Deng X, Li Y, Cai X, et al. Detectable 2019-nCoV viral RNA in blood is a strong indicator for the further clinical severity. *Emerg Microbes Infect*. (2020) 9:469. doi: 10.1080/22221751.2020.1732837
11. Chen Y, Chen L, Deng Q, Zhang G, Wu K, Ni L, et al. The presence of SARS-CoV-2 RNA in feces of COVID-19 patients. *J Med Virol*. (2020) 92:833–40. doi: 10.1002/jmv.25825
12. Kim JY, Ko JH, Kim Y, Kim YJ, Kim JM, Chung YS, et al. Viral load kinetics of SARS-CoV-2 infection in first two patients in Korea. *J Korean Med Sci*. (2020) 35:e86. doi: 10.3346/jkms.2020.35.e86
13. Kam KQ, Yung CF, Cui L, Lin Tzer Pin R, Mak TM, Maiwald M, et al. A well infant with coronavirus disease (2019) (COVID-19) with high viral load. *Clin Infect Dis*. (2020) 71:847–9. doi: 10.1093/cid/ciaa201
14. Lescure FX, Bouadma L, Nguyen D, Parisey M, Wicky PH, Behillil S, et al. Clinical and virological data of the first cases of COVID-19 in Europe: a case series. *Lancet Infect Dis*. (2020) 20:P697–706. doi: 10.1016/S1473-3099(20)30200-0
15. Lo IL, Lio CF, Cheong HH, Lei CI, Cheong TH, Zhong X, et al. Evaluation of SARS-CoV-2 RNA shedding in clinical specimens and clinical characteristics of 10 patients with COVID-19 in Macau. *Int J Biol Sci*. (2020) 16:1698–1707. doi: 10.7150/ijbs.45357
16. Pan Y, Zhang D, Yang P, Poon LLM, Wang Q. Viral load of SARS-CoV-2 in clinical samples. *Lancet Infect Dis*. (2020) 20:411–12. doi: 10.1016/S1473-3099(20)30113-4
17. To KK, Tsang OT, Chik-Yan Yip C, Chan KH, Wu TC, Chan JMC, et al. Consistent detection of 2019 novel coronavirus in saliva. *Clin Infect Dis*. (2020) 71:841–3. doi: 10.1093/cid/ciaa149
18. To KK, Tsang OT, Leung WS, Tam AR, Wu TC, Lung DC, et al. Temporal profiles of viral load in posterior oropharyngeal saliva samples and serum antibody responses during infection by SARS-CoV-2: an observational cohort study. *Lancet Infect Dis*. (2020) 20:565–74. doi: 10.1016/S1473-3099(20)30196-1
19. Wang W, Xu Y, Gao R, Lu R, Han K, Wu G, et al. Detection of SARS-CoV-2 in different types of clinical specimens. *JAMA*. (2020) 323:1843–4. doi: 10.1001/jama.2020.3786
20. Young BE, Ong SWX, Kalimuddin S, Low JG, Tan SY, Loh J, et al. Epidemiologic features and clinical course of patients infected with SARS-CoV-2 in Singapore. *JAMA*. (2020) 323:1488–94. doi: 10.1001/jama.2020.3204
21. Yu F, Yan L, Wang N, Yang S, Wang L, Tang Y, et al. Quantitative detection and viral load analysis of SARS-CoV-2 in infected patients. *Clin Infect Dis*. (2020) 71:793–8. doi: 10.1093/cid/ciaa345
22. Zhang W, Du RH, Li B, Zheng XS, Yang XL, Hu B, et al. Molecular and serological investigation of 2019-nCoV infected patients: implication of multiple shedding routes. *Emerg Microbes Infect*. (2020) 9:386. doi: 10.1080/22221751.2020.1729071
23. Wölfel R, Corman VM, Guggemos W, Seilmaier M, Zange S, Müller MA, et al. Virological assessment of hospitalized patients with COVID-2019. *Nature*. (2020) 581:465–9. doi: 10.1038/s41586-020-2196-x
24. Xiao F, Tang M, Zheng X, Liu Y, Li X, Shan H. Evidence for gastrointestinal infection of SARS-CoV-2. *Gastroenterology*. (2020) 158:1831–3.e3. doi: 10.1101/2020.02.17.20023721
25. Zou L, Ruan F, Huang M, Liang L, Huang H, Hong Z, et al. SARS-CoV-2 viral load in upper respiratory specimens of infected patients. *N Engl J Med*. (2020) 382:1177–9. doi: 10.1056/NEJMc2001737
26. Yang Y, Yang M, Shen C, Wang F, Yuan J, Li J, et al. Original article Evaluating the accuracy of different respiratory specimens in the laboratory diagnosis and monitoring the viral shedding of 2019-nCoV infections. *MedRxiv [Preprint]*. (2020). doi: 10.1101/2020.02.11.20021493
27. Liu Y, Yang Y, Zhang C, Huang F, Wang F, Yuan J, et al. Clinical and biochemical indexes from 2019-nCoV infected patients linked to viral loads and lung injury. *Sci China Life Sci*. (2020) 63:364. doi: 10.1007/s11427-020-1643-8
28. Qiu L, Liu X, Xiao M, Xie J, Cao W, Liu Z, et al. SARS-CoV-2 is not detectable in the vaginal fluid of women with severe COVID-19 infection. *Clin Infect Dis*. (2020) 71:813–17. doi: 10.1093/cid/ciaa375
29. Gautret P, Lagier JC, Parola P, Hoang VT, Meddeb L, Mailhe M, et al. Hydroxychloroquine and azithromycin as a treatment of COVID-19: results of an open-label non-randomized clinical trial. *Int J Antimicrob Agents*. (2020) 56:105949. doi: 10.1016/j.ijantimicag.2020.105949
30. Liu F, Xu A, Zhang Y, Xuan W, Yan T, Pan K, et al. Patients of COVID-19 may benefit from sustained lopinavir-combined regimen and the increase of eosinophil may predict the outcome of COVID-19 progression. *Int J Infect Dis*. (2020) 95:P183–91. doi: 10.1016/j.ijid.2020.03.013
31. Meng J, Xiao G, Zhang J, He X, Ou M, Bi J, et al. Renin-angiotensin system inhibitors improve the clinical outcomes of COVID-19 patients with hypertension. *Emerg Microbes Infect*. (2020) 9:757. doi: 10.1080/22221751.2020.1746200
32. Rothe C, Schunk M, Sothmann P, Bretzel G, Froeschl G, Wallrauch C, et al. Transmission of 2019-nCoV infection from an asymptomatic contact in Germany. *N Engl J Med*. (2020) 382:970–71. doi: 10.1056/NEJMc2001468
33. Sjödin H, Wilder-Smith A, Osman S, Farooq Z, Rocklöv J. Only strict quarantine measures can curb the coronavirus disease (COVID-19) outbreak in Italy 2020. *Euro Surveill*. (2020) 25:2000280. doi: 10.2807/1560-7917.ES.2020.25.13.2000280
34. Lau H, Khosrawipour V, Kocbach P, Mikolajczyk A, Schubert J, Bania J, et al. The positive impact of lockdown in Wuhan on containing the COVID-19 outbreak in China. *J Travel Med*. (2020) 27:taaa037. doi: 10.1093/jtm/taaa037
35. Chan PK, To WK, Ng KC, Lam RK, Ng TK, Chan RC, et al. Laboratory diagnosis of SARS *Emerg Infect Dis*. (2004) 10:825. doi: 10.3201/eid1005.030682
36. van Dorp L, Acman M, Richard D, Shaw LP, Ford CE, Ormond L, et al. Emergence of genomic diversity and recurrent mutations in SARS-CoV-2. *Infect Genet Evol*. (2020) 83:104351. doi: 10.1016/j.meegid.2020.104351
37. Phan T. Genetic diversity and evolution of SARS-CoV-2. *Infect Genet Evol*. (2020) 81:104260. doi: 10.1016/j.meegid.2020.104260
38. Khailany RA, Safdar M, Ozaslan M. Genomic characterization of a novel SARS-CoV-2. *Gene Rep*. (2020) 19:100682. doi: 10.1016/j.genrep.2020.100682
39. Cagliani R, Forni D, Clerici M, Sironi M. Computational inference of selection underlying the evolution of the novel coronavirus, severe acute respiratory syndrome coronavirus 2. *J Virol*. (2020) 94:e00411-20. doi: 10.1128/JVI.00411-20
40. Fung TS, Liu DX. Human coronavirus: host-pathogen interaction. *Annu Rev Microbiol*. (2019) 73:529–57. doi: 10.1146/annurev-micro-020518-115759
41. Yu F, Du L, Ojcius DM, Pan C, Jiang S. Measures for diagnosing and treating infections by a novel coronavirus responsible for a pneumonia outbreak originating in Wuhan, China. *Microbes Infect*. (2020) 22:74–9. doi: 10.1016/j.micinf.2020.01.003

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Communication

ORIGINAL RESEARCH
published: 20 August 2020
doi: 10.3389/fcomm.2020.00058

COVID-19, Authoritarian Neoliberalism, and Precarious Migrant Work in Singapore: Structural Violence and Communicative Inequality

*Mohan Jyoti Dutta**

Center for Culture-Centered Approach to Research and Evaluation, Massey University, Wellington, New Zealand

Drawing upon an ongoing ethnography with low-wage migrant workers in Singapore, this article builds on the theoretical framework of the culture-centered approach (CCA) to explore the experiences of the workers amid COVID-19 outbreaks in dormitories housing them. The CCA foregrounds the interplays of communicative and material inequalities, suggesting that the erasure of infrastructures of voices among the margins reproduces and circulates unhealthy structures that threaten the health and well-being of the working classes. The voices of the low-wage migrant workers who participated in this study document the challenges with poor housing, poor sanitation, and food insecurity that are compounded with the absence of information and voice infrastructures. Amid the everyday threats to health and well-being that are generated by neoliberal reforms across the globe, the hyper-precarious conditions of migrant work rendered visible by the trajectories of COVID-19 call for structurally transformative futures that are anchored in the voices of workers at the margins of neoliberal economies.

Keywords: low-wage migrant work, COVID-19, Singapore, authoritarianism, outbreak inequality, extreme neoliberalism, culture-centered approach, migration

OPEN ACCESS

Edited by:
Shaunak Sastry,
University of Cincinnati, United States

Reviewed by:
Simon Springer,
The University of Newcastle, Australia
Benjamin R. Bates,
Ohio University, United States

***Correspondence:**
Mohan Jyoti Dutta
m.j.dutta@massey.ac.nz

Specialty section:
This article was submitted to Health Communication, a section of the journal Frontiers in Communication

Received: *11 May 2020*
Accepted: *06 July 2020*
Published: *20 August 2020*

Citation:
Dutta MJ (2020) COVID-19, Authoritarian Neoliberalism, and Precarious Migrant Work in Singapore: Structural Violence and Communicative Inequality. Front. Commun. 5:58. doi: 10.3389/fcomm.2020.00058

INTRODUCTION

Shameem had traveled to Bangladesh 11 years ago. He has lived in a wide range of accommodations in Singapore. He shares how when he first came, he lived in a container. He shares that life in the container was challenging. The conditions were unlivable. He shares that he shared the space in the container with rodents and cockroaches. When he compares life in the container with life in the dormitory he lives in now, not much has changed in the decade. He voices how workers are piled on "top of each other" in the room, with little room to move. He shares that he has felt all along that an outbreak such as the one we are witnessing now was waiting to happen. He shares anger and despair at the knowledge that the outbreak could have been prevented. But no one really cared, he points out. He also states that having decent housing is the right of the worker, and the worker does not need alms. The plight of the worker is not visible to anyone in Singapore (fieldnotes, April 25, interview).

Singapore's authoritarian techniques of disciplining labor form the infrastructures for propping up, circulating, and generating profits from the "Singapore model" of extreme neoliberalism. I define extreme neoliberalism as the free market ideology pushed beyond its organizing limits, with

the structuring of the state as an authoritarian instrument of control that silences and co-opts worker collectivization, generating precarity while simultaneously deploying the logics of business-friendliness to enable the mobility of capital across spaces/borders[1]. Extreme neoliberalism is held up by the work of communicative infrastructures that simultaneously erase the symbolic registers for comprehending the deep inequalities produced by the neoliberal state through the combination of authoritarian control and accelerated propaganda. Singapore's authoritarian neoliberalism, as an assemblage of "coercive legal, institutional, and policy processes" (Bruff, 2014, p. 112) to govern by moralizing the individual, family, and community, draws upon a wide array of techniques of surveillance and violence incorporated into everyday life. Springer (2015) theorizes violent neoliberalism, referring to the "the kaleidoscope of violence that is intercalated within neoliberalism's broader rationality of power," (p. 12) arguing that violence constitutes the deep inequalities produced by neoliberalism and is in turn, constituted by these inequalities. He suggests that this violence is both exceptional, appearing "to fall outside of the rule, usually by being so intense in its manifestation," and "in a dialectic relationship with exemplary violence, or that violence which constitutes the rule" (p. 2).

This interplay of violence and authoritarianism is incessantly normalized in extreme neoliberalism through the everyday acts of "communicative inversions" (Dutta, 2015), reversals of materiality through symbolic productions, and strategic communicative erasures. As both a role model and pedagogue of extreme neoliberalism since the 1980s, Singapore has continually experimented with and perfected the techniques of authoritarian repression, exploitation of labor, and accumulation of primitive capital. It has invented the statecraft of disciplining labor and silencing dissent as model governmentality, while turning itself into the Asian gateway for transnational capital. The technologies and techniques of extreme neoliberalism are held up by a reputational economy that projects the account of a hyper-efficient state celebrated as the model of development, embodied in its "smart city" imaginary/propaganda leapfrogging from the "Third world to the first" at the frontiers of global capitalist expansion. This "smart city" template is circulated across nation states as a model for growth-driven development punctuated by profiteering across global networks of capital while simultaneously disseminating a pedagogy of authoritarian techniques of disciplining the margins (Tan, 2012; Dutta and Kaur-Gill, 2018; Dutta, 2019a,b). The extreme inequalities, alongside normative practices of disciplining workers, critics, and activists form a mobile infrastructure of the "Singapore model," to be copied into state formations elsewhere, "rolling out" a police state to facilitate and catalyze accelerated capital accumulation (Tansel, 2017; Bruff and Tansel, 2019). Singapore's "smart" governance assembles a collection of laws, surveillance technologies, controls over institutions and civil society, and police interventions aimed at repression while simultaneously "rolling back" the welfare-based role of the state. At the heart of the "smart city" infrastructure of Singapore is the exploited labor of low-wage migrant workers, accompanied by the strategic deployment of a range of tactics of violence to erase migrant worker voices and invisibilize migrant worker bodies (see for instance Kaur et al., 2016; Yea, 2017).

Migrating from Bangladesh, India, Thailand, Malaysia, and China, low-wage migrant workers labor in precarious positions in Singapore, building the infrastructures of Singapore's neoliberal economy. Toiling in transient conditions without access to pathways of citizenship, without labor rights, and without the access to communication infrastructures for voicing the challenges to their health and well-being, low-wage migrant workers live in a climate of fear, amidst systemic threats to their employment, health, and well-being (Dutta, 2017a,b; Yea, 2017; Yea and Chok, 2018). The everyday struggles for migrant health in Singapore are constituted amidst a climate of authoritarian state management that produces worker precarity to facilitate capitalist extraction. Structural violence, reflected in the denial of the fundamental needs of everyday health and well-being, is constituted amidst the cultivated climate of fear and erasure (Dutta, 2017a,b).

The COVID-19 pandemic renders visible the deep inequalities entrenched in the political economy of Singapore, with the largest number of COVID-19 infections among low-wage male migrant workers. Singapore, celebrated as the "gold standard" and held up by World Health Organization (WHO) as a model response in the early part of the pandemic, emerged as the site of large-scale COVID-19 outbreaks in migrant worker dormitories. Why did a city-state paraded as an exemplar of efficient expertise-driven neoliberal management of the pandemic, replete with its methods of contact tracing, quarantining, and keeping borders open, turn into a site of large-scale pandemic outbreak, moving at the top of the chart of COVID-19 infections in Southeast Asia by early May? In this article, I apply the theoretical framework of the culture-centered approach (CCA) to argue that inherent in Singapore's model of authoritarian neoliberalism is the infrastructure of technocratic management that erases the voice of precarious migrant laborers. The management of migrant labor is carried out through repressive strategies of disciplining, and this constitutes the backdrop of the COVID-19 outbreaks in migrant worker dormitories in Singapore. Drawing on my ethnographic work with low-wage migrant workers in Singapore, I suggest that the initial control of the outbreak through technocratic techniques of generating efficiency exists in continuity with the systematic erasure of the voices of migrant workers and the vast power inequalities amidst which low-wage migrant workers live their lives. I explore the interplays between structural violence and communicative inequality amidst the outbreak in migrant worker dormitories based on my ethnographic fieldnotes, in-depth interviews, a digital ethnography, and a survey.

[1]Singapore is consistently ranked as one of the best places to do business by the World Bank's *Doing Business Report*. Simultaneously, consider the ranking of Singapore toward the bottom in the Oxfam *Commitment to reducing* inequality report. As a destination for transnational capital, Singapore continually works on the production of a disciplined workforce, low public sector spending, and low corporate taxes. Since the 1980s, Singapore's public sector spending has continually declined (Low, 2014). The state's expenditures on healthcare and social services are among the lowest in developed economies globally (Rahim and Barr, 2019).

CULTURE-CENTERED APPROACH

The CCA locates health meanings at the intersections of culture, structure, and agency (Dutta, 2004a, 2008, 2017a,b; Dutta and Jamil, 2013). Culture reflects the dynamic interaction between shared meanings and contexts, drawing upon and shaping the shared values, beliefs and practices in everyday life. Structure taps into the patterns of distribution of social, material, political, and economic resources of health and well-being (Dutta and Basu, 2008; Sastry et al., 2019). Agency is the human capacity to make sense of and negotiate the everyday contexts of health and well-being (Dutta, 2004a, 2017a,b; Dutta and Jamil, 2013; Bates et al., 2019). Anchoring the meanings of COVID-19 constructed by low-wage migrant workers in Singapore amidst the interplays of culture, structure, and agency opens up the spaces of theorizing rooted in migrant worker voices, working in solidarity with these voices to co-create praxis (Dutta et al., 2017; Jamil and Kumar, 2020).

In the conceptual framework put for by the CCA, communicative inequalities (inequalities in the distribution of communicative resources for information and voice) are intricately intertwined with structural inequalities, or inequalities in the distribution of material resources (Dutta, 2004a, 2007, 2008). The erasure of the voices of low-wage migrant workers from discursive spaces is intertwined with the structural violence workers experience. Therefore, the work of co-creating infrastructures for voice is embedded in material inequalities, and simultaneously offers an anchor to transforming these material inequalities.

The interplays of communicative and structural inequalities constitute the inequalities in health outcomes (Newman et al., 2014; Dutta et al., 2017). These inequalities in health outcomes, produced by extreme neoliberal policies, are likely to be exacerbated in the context of COVID-19. Hegemonic health communication approaches erase these structural contexts of health, instead developing individualized behavior change solutions promoting changes in individual attitudes and beliefs. Even as structural determinants of health are offered lip service in hegemonic health communication interventions, the behavioral recommendations focus on the individual. Interventions addressing health disparities primarily take the forms of culturally sensitive campaigns incorporating relevant cultural characteristics into behavior change messages or technology-based solutions. Specifically in the context of COVID-19, hegemonic health communication solutions target individual behavior, locating the problem in the individual, placing the responsibility of mask wearing, social distancing, hand washing, eating healthy, and staying home on the individual[2]. The CCA inverts this individualized reductionism through the co-creation of communication infrastructures with community voices at the margins. The discursive registers put forth by the voices of those at the margins foreground the structural features of neoliberal societies as the sites of transformative organizing and social change.

Building on a growing line of existing research that connects health to the precarity of work in global processes of labor flow (Dutta and Jamil, 2013; Kaur et al., 2016; Dutta, 2017a,b; Yea, 2017), the CCA suggests that the structural contexts of immigrant health are rooted in the erasure of migrant voices. Voices of low-wage migrants at the margins of neoliberal economies foregrounds meanings amid these structures, suggesting strategies for health communication that responds to these overarching structures of health and migration. It seeks to co-create infrastructures of listening to the voices of migrant construction workers in Singapore, seeking to disrupt the ongoing forms of erasure that constitute the organizing of migrant spaces (Dutta, 2004a,b, 2008; Dutta and Jamil, 2013; Dutta and Kaur-Gill, 2018).

NEOLIBERAL SINGAPORE AND STRUCTURES OF LOW-WAGE MIGRATION

Singapore, with its role as the frontier of transnational capital's cultivation of and investments into ever-expanding Asian markets, as well as the infrastructure of neoliberal pedagogies of the future (consider the powerful presence of Singapore elites as interlocutors and mediators in World Economic Forum conversations on elite responses), constitutes a key register of extreme neoliberalism. Singapore's extreme neoliberalism incorporates techniques of violence and disciplining into the authoritarian state as the organizing structure for capitalist accumulation. The model of state capitalism explicitly organizes the state not only as a "custodian of capital accumulation" (Tansel, 2017, p. 4), but also as a pivotal player in the processes of extraction and exploitation. The authoritarian state as the primary agent of "neoliberalizing violence" (Springer, 2015) incorporates into its structures mechanisms of surveilling, disciplining and policing the abandoned "other." It thrives on the production and circulation of propaganda that props up its model of economic development, pitched as the "Singapore model" (see Dutta, 2018a,b; Dutta et al., 2019). Underlying the propaganda of the "Singapore model" is an entire infrastructure of communicative devices that project "hubs," "knowledge centers," "dialogues," and "expos" that are continually at work, seeding, promoting, and circulating the model of free market economics, narrated in the language of Asian values (Dutta, 2018a,b, 2019a,b). The carefully crafted public relations infrastructure of Singapore continually manufactures the narrative of "from third world to first." Singapore's communicative capital thrives on the erasure of the exploitation and extraction that constitute its global positioning as the frontiers of transnational capitalist expansion into Asia (Dutta, 2019a,b). This communicative capital, reflected in Singapore's positioning as a hub for the creative and digital industries accompanied by the numerous public relations campaigns run by the state positioning Singapore as the city of the futures, is sustained through the systematic deployment of techniques

[2]Consider for instance the campaign run by the World Health Organization (WHO), #HealthyAtHome, with recommendations of preventive behaviors, putting forth and circulating the hegemony of behavior change (*https://www.who.int/campaigns/connecting-the-world-to-combat-coronavirus/healthyathome?gclid$=$EAIaIQobChMIheHjP6M6gIVliQrCh2djwWDEAAYASAAEgJBfDBwE*).

of disciplining that manage, surveil, and silence the poor and working classes.

The authoritarian techniques of control and discipline that form the backbone of Singapore's neoliberalism are packaged into the brand of "Asian values," then sold as a destination solution to these othered elsewhere of Asia (Tan, 2012; Juego, 2018). The erasure of the exploitation of low-wage migrant workers from Bangladesh and India is necessary to crafting the seduction of the "Singapore model" as hyper-efficient governance for these backward Asia's. The seduction of the "Singapore model" is sold as a "smart city" imaginary of the future to these other Asia's, thus generating news sites of extraction of profiteering for Singapore-based corporations (consider for instance the market opportunities opened up for Singapore-based corporations in India's "Smart City" projects pursued aggressively). The narrative of lifting large parts of Asia's backward spaces out of poverty strategically obfuscates the poor working conditions, the absence of minimum wage, the lack of opportunities for collectivization, and the absence of worker rights for low-wage migrant workers in Singapore. The neoliberal narrative of the trickle-down effect works simultaneously through the inversion of materiality and the erasure of worker voices. The authoritarian strategies of controlling protest and organizing work hand-in-hand to erase opportunities for worker articulations of labor rights.

The narrative of the "Singapore model," storying an account of a strong developmental state that actively fosters development through its strong interventions is manufactured as a seduction for urban development across Asia (Dutta, 2018a,b). The model promises a policy mix that combines authoritarian management with state-led development (Pow, 2014). The strong state-based interventionist model, evident in the state-run corporations and the climate of authoritarian repression of collective organizing form the architectures of Singapore's extreme neoliberalism. The model of "hybrid development" (Rahim and Barr, 2019) serving the frontiers of capitalist expansion strategically crafts an account of "Asian values" to render as Asian forms of repression and disciplining that serve the expansionary interests of transnational capital. The ideology of "Asian values" strategically manufactured, planted and circulated by Singapore's ruling elite, concocts a mixture of narratives of meritocracy, pragmatism, and communitarianism into brand Singapore that drives the nation state's political economy. In the latest iteration of techno-capitalist futures, the "Singapore model" punctuates the story of the "smart city," with futuristic imaginaries of creative capital, digital participation, and sustainable technologies for growth (Kong, 2018; Kong and Woods, 2018). An array of policies of city making, projected as smart policies work together to craft this imaginary of futures. Underlying the mobility of the techno-futuristic appeal of the "Singapore model" lies the systemic exploitation of precarious workers that materializes the technologies of clean, urban planning. Voices of low-wage migrant workers are silenced with a legal framework that criminalizes migrant worker organizing, censors migrant worker protest, and strongly regulates migrant worker presence in spaces of public participation through technologies of surveillance and police control.

Migrant Health

Low-wage migrant workers working in the construction, shipping, building, and cleaning industries in Singapore, form the textures of global flow of labor amidst extreme neoliberal policies. Migrant workers negotiate their health amidst global structures of capitalist extraction (Dutta, 2017a,b). The theorizing of health of low-wage migrant workers in Singapore ought to be situated amidst its "smart city model" marketed globally as a model of labor extraction through techniques of authoritarian disciplining that enable capitalist expansion. That the material architecture of smart city Singapore is built on the extracted labor of low-wage migrant workers is communicatively erased, using the tools of "trickle down" narratives and erasing the bodies of low-wage migrant workers from Singapore's smart urbanisms. Low-wage migrant workers in Singapore often work in "dirty, dangerous, and difficult" jobs, supported on short-term work permits, without labor protections and without the pathways of mobility into citizenship (Baey and Yeoh, 2015; Bal, 2015).

Low-wage contract-based migrant workers in Singapore perform precarious work, work that has "limited social benefits and statutory entitlements, job insecurity, low wages, and high risks of ill health" (Vosko, 2006, p. 4). The everyday work experiences of low-wage migrant workers are constituted amidst vast imbalances in distribution of power, with the control over their short-term work permits held by the employer (Yea and Chok, 2018). Amidst restrictive migration laws that promote temporariness and preclude pathways of mobility into citizenship, complex, and interconnected webs of brokerage constitute the tenuous conditions of low-wage migrant labor in Singapore (Lindquist et al., 2012; Baey and Yeoh, 2015, 2018). Lewis et al. (2015) depict these conditions of low-wage migrant labor as "hyper precarious," reflected in "deportability, risk of bodily injury coupled with restricted access to healthcare, and transactional relationships" (p. 593). Hyper precarious work is marked by the absence of protections, and a fundamental condition of "unfreedom" (Yea, 2017; Yea and Chok, 2018). The linkages of brokerage, materialized in the form of recruitment, training, and travel agencies, impose significant front-end investments on low-wage contract-based migrant workers, which are often secured by going into debt, selling the limited ancestral land, or selling household possessions The hyper-precarity of low-wage contract-based migrant work in Singapore is further exacerbated by the individualization of the risks on the worker, with the absence of systemic infrastructures for workers to address their labor-related needs, the absence of state-based infrastructures directly accessible to workers, and the absence of clear policy oversight that holds the employers, dormitories, and caterers accountable.

The systematic erasure of the voices of low-wage migrant workers is accompanied by communicative inversions, the turning-on-its-head of materiality through techniques of strategic communication (Dutta, 2016). For instance, the materiality of migrant worker precarity is inverted by communicative devices that project Singapore as the gateway for upward mobility for the dark-skinned masses of the Third World. This work of communicative inversion is propelled by a

racist communicative architecture that needs the backwardness of neighboring Asia's (Bangladesh, India) to construct Singapore in the imagery of Whiteness. Low-wage migrant work is thus narrated as upward mobility, forming the rhetorical arsenal of trickle-down economics through migration that catalyzes economic mobility elsewhere in the Third World. In this ideology, the migration of low-wage migrant workers to Singapore as a hub of Asian capital uplifts individuals, households, communities, and nations that form Asia's other into the networks of mobility.

METHOD

This manuscript reports on two distinct phases of data gathering, nested within a broader ongoing culture-centered intervention seeking to co-create infrastructures for health and well-being among low-wage migrant workers in Singapore (Dutta, 2018a). Following an immersed 6 month ethnography with low-wage migrant workers in Singapore conducted in 2008, between 2012 and 2018, an advisory group of low-wage migrant workers that had been formed as part of the ongoing culture-centered intervention developed by the Center for Culture-centered Approach to Research and Evaluation (CARE) sought to co-create everyday solutions of health and well-being, resulting in a national-level health campaign, "Respect our rights" seeking to transform the unhealthy structures of migrant work (see https://www.facebook.com/Migrant-Workers-Rights-SG-1557463061204402/).

When the COVID-19 outbreaks emerged in migrant worker dormitories, the advisory group sought to co-create COVID-19-related health solutions addressing the outbreak in migrant worker dormitories. They shaped the design of the COVID-19-specific study, as well as the process of making sense of the emergent data. Given the longitudinal nature of the project, three separate human ethics approvals supported it. The latest round of data gathering reported here was deemed to be low-risk following university ethics procedures. Given the precarity of low-wage migrant workers amidst Singapore's authoritarian surveillance structures, multiple steps were taken to anonymize worker identity, including transcribing the interviews immediately, erasing the audio files immediately after transcription, and not attaching identifiers to narrative accounts.

The findings reported here draw on two phases of data gathering amidst COVID-19. In the first phase, the data were gathered from on an ongoing digital ethnography (87 h of participant observation) conducted in spaces where low-wage migrant workers participate online and 47 semi-structured interviews conducted with low-wage migrant workers between April 7, 2020 and April 30, 2020. The participant observation included making detailed notes of online interactions, coding issue-specific articulations, negotiations of power, as well as the strategies for communicative negotiations. The participants for the interviews were identified using snowball sampling, guided theoretically by the principle of co-creating the "margins of the margins" (Dutta, 2018a). The interviews were conducted in Bengali, mix of Bengali and English, or English, depending on the level of comfort and preference of the participant. Data analysis in the first phase was carried out through line-by-line coding of the 47 interviews, followed by the organizing of the codes into broader themes. The initial themes emergent from the analysis were shared with the advisory group, who made sense of the themes through their lived experiences amidst COVID-19. The advisory group determined the key findings to be reported based on the consideration of the immediate challenges they have been experiencing amidst COVID-19[3].

In the second phase, a survey was conducted. The advisory group of migrant workers, drawing upon the emergent themes in the interviews, co-constructed with the research team a survey exploring the challenges to health and well-being amidst COVID-19. This article incorporates descriptive statistics from the survey. The initial survey was pilot tested among 10 workers. Participants were recruited through snowball sampling, with the link to the survey circulated in networks of low-wage migrant workers. In addition, migrant workers were recruited through social networks and the survey was administered over phone. The sample ($n = 101$ usable responses, from 106 participants) comprises predominantly Bangladeshi migrant workers, with representation by a smaller number of Indian workers. The sample does not include Chinese workers. Workers from Bangladesh and India constitute some of the lowest rungs of low-wage migrant work in Singapore.

The phased three-pronged research approach combining digital ethnography, semi-structured interviews, and survey enabled validation, offering a framework for examining the convergence of the findings. Moreover, the longitudinal process of sense-making of the data conducted by the advisory group of migrant workers further strengthened the validity of the analysis. Throughout the process of making sense of the data, the advisory group members noted how their experiences with the challenges to health and well-being in the everyday contexts of life in Singapore pre-COVID-19 converged with the emergent challenges amidst COVID-19. Therefore, to further validate the findings, the data gathered from the participant interviews conducted in the context of COVID-19 were placed in conversation with 157 in-depth interviews that were conducted by CARE since 2012, anchored in the tenets of the CCA (although the narrative excerpts included here do not report from the 2012–2018 period). These earlier in-depth interviews, guided by a continually transforming advisory group, had identified the structural contexts of work and living that constitute the everyday challenges to health and well-being of low-wage migrant workers

[3]The findings reported in this manuscript formed the basis of two white papers reported by CARE, seeking to intervene into the COVID-19 state response. They were extensively covered in national and global media (*https://www.theguardian.com/world/2020/apr/23/singapore-million-migrant-workers-suffer-as-covid-19-surges-back*), drawing attention to the poor living conditions experienced by low-wage migrant workers in Singapore. These findings along with advocacy work carried out by activists fostered a public opinion climate amidst which the state introduced fundamental transformations to the housing arrangements for low-wage migrant workers in Singapore (*https://www.todayonline.com/singapore/new-dorms-better-standards-be-built-100000-foreign-workers-coming-years-lawrence-wong?fbclid$=$IwAR3T8JWaNW-~Vu33KBdnG71UrxHB85oVWeCC6InRgKfvd29LAdQBZg2EKmQA*).

in Singapore. For instance, the challenges with housing and food as integral to the health and well-being of migrant workers.

FINDINGS

This article specifically draws on findings that foreground the structural contexts of health, further exacerbated amidst COVID-19. The ethnography, interview, and survey data point to the structures of housing, the absence of sanitation infrastructures, and the experiences of food insecurity as the contexts within which health and well-being are negotiated. The structural violence that is exacerbated amidst COVID-19 is placed amidst symbolic violence, the absence of infrastructures of information and voice articulated by the low-wage migrant workers. These interactions between communicative and structural violence form the overarching infrastructures of poor health and well-being, within which low-wage migrant workers voice the everyday challenges to mental health amidst COVID-19.

Structures of Housing

The poor condition of housing is a key theme in the ongoing ethnographic work. In creating a framework for understanding the challenges to health and well-being, low-wage migrant workers had noted in the first round of advisory group meetings held by the CARE research team in 2013 that the cramped conditions within which they live their lives threaten their health and well-being, foregrounding the living conditions as an important anchor to worker health and well-being. This theme of poor housing conditions was narrated by participant 32[4], who noted "How can a worker be healthy when there are so many of us in one room? There is no space to breathe, and everyone is stacked together into the room." For participant 67, "I just can't move around in the room." Another participant suggests that the air circulation in the room is poor, "I feel I can't breathe. This is my biggest health challenge. There is a person on the bed above me." Yet another participant noted, "The room leaves no space to stretch or even rest after a hard day at work. When I return home, I am tired. Then there are so many brothers all together. This makes me sick." Another participant noted, "I can't breathe. The air is stale and there is smell in the room." The participants in our research narrate the ways in which the overcrowding in the living arrangements turns into experiences of stress and anxiety. Notes a participant, "I have worked on so many buildings. Many of them are big hotels. But this is how we live. All crowded together in these rooms with nowhere to go."

They also suggest that with many workers in a room, there are often conflicts, which further exacerbate the feelings of stress experienced by them. Notes participant 113, "the conflict is often about the space. There are so many challenges with space, about where to keep things, when to turn on the lights, when to speak with family. All of this has to be figured out." Negotiating their work rhythms, participants suggest that their sleep patterns are affected because of different work schedules. This in turn significantly affects their health and well-being, and also contributes to accidents in the workplace. The participants in our research highlight ongoing challenges with privacy in the rooms. Notes a participant, "With so many people in the room, there is no privacy." With 20 migrant workers in a room in many instances, participants note that they are unable to communicate on the phone, move freely in their rooms, and have a sense of peace. This lack of privacy results in conflicts among workers in the rooms, and adversely affects the mental health and well-being of workers. Noted a participant, "I can't sleep at night because some of the brothers in the room wake up early in the morning. This results in a sense of being tired all day." Another participant voiced, "How can anyone rest when there are so many of us." Another participant pointed out, "I have to go outside and walk around in the hallway if I want to talk to my wife. There are often money matters that we are discussing." The sense of not having privacy, often with 20 workers in a room, adversely impacts the sense of health and well-being.

Amidst these everyday challenges to health and well-being constituted amidst the architecture of the rooms, participants point to the sense of feeling depressed, "I didn't think before coming here I will have to live like this. In (referring to home), we have open fields, and open air. When I came here and looked at how I have to live, I became sad." Multiple participants refer to feelings of depression when discussing their living arrangements. This feeling of depression is constituted amidst articulations of not knowing how to change these conditions (more on this later).

Making sense of this condition of overcrowding, a participant observes, "The boss does not care. The dorm owner makes as much money from the worker as he can." Although participants point to a wide range of living arrangements, for a large number of them, the usual living arrangement is in a dormitory, with between 15 and 20 workers in a room. For another participant, "The workers are put in like in a jail. There is no room to move." Participants often wondered about the approval process for building these arrangements and what the regulatory guidelines were, "The answer for why, our dormitory authority, or dormitory approval authority only know, who give them the approval to arrange 20 person." A participant pointed to absence of oversight, "Even the place where I am staying, some time they bring in even more workers." These overcrowded arrangements affect the health and well-being of workers.

Housing and the Limits to Behavior Change

The concerns about over-crowded rooms becomes salient amidst the COVID-19 outbreaks in the dormitories housing the workers. The narratives locate the one-meter physical/social distancing policy amidst the crowded living conditions, suggesting that the recommendation to maintain one-meter distance does not make sense because of the very nature of worker housing. Noted one participant, "For me, I exactly don't know my room size, but I feel that 10 person also maximum in my room, but they keep 20 person." Another noted, "They keep bringing in more workers into the room. There is no space to move even." In the midst of the pandemic, participants point to workers being moved, often without clear communication, and often without

[4]In this project, participants felt that their identities needed to be protected as they were voicing aspects of their livelihood that potentially could pose challenges to the tenability of their employment, work permit, and safety in Singapore. The co-constructive process with the advisory group resulted in.

addressing the crowded conditions. Referring specifically to the COVID-19 outbreak, a participant noted "How can a worker follow 1 m distance? The room has 20 people." Here's another excerpt from an interview, "Now, there are many of us in the room all day every day. Because I cannot go outside, I am staying inside the room. Everyone is doing this. So compared to regular times, now there are even more workers in the room throughout the day." Another participant noted, "They are saying you need to do those things, washing hand and not go outside together. How can we follow one meter distance when there are so many workers in a room."

This structural limitation on following the one-meter social/physical distancing guideline is reiterated in the survey. For a large majority of low-wage migrant workers in Singapore, self-reported practice of one-meter distance in the dorms is unlikely within the housing infrastructures. In response to the statement, "I am able to follow one meter rule of social distance where I stay," 38.4% "strongly disagreed," 28.3% "disagreed," and 9.1% "somewhat disagreed" (see **Figure 1**).

Most of the workers attributed this inability to follow the one-meter rule to the cramped conditions in the dormitory. In response to the statement, "I can't follow the one-meter rule of social distance because of cramped conditions in the dormitory," 12% "somewhat agreed," 31% "agreed," and 35% "strongly agreed" (see **Figure 2**).

Structures of Sanitation

Inadequate sanitation has consistently emerged as a theme in our ethnographic solidarities with low-wage migrant workers in Singapore. In advisory group meetings, workers have often narrated the poor toilet facilities in their spaces of living as well as at work sites across Singapore. Amidst the COVID-19 outbreak in dormitories, worker voices foregrounded the ways in which the unclean dormitory conditions posed risks to their health and well-being.

Unclean Dormitory Arrangements

Tied to the concerns about overcrowding are the articulations of unclean dormitory arrangements. The participants note that overcrowding often leads to unclean dormitory arrangements, with the usage increasing because of the number of workers that are staying in their rooms. Throughout the period of ethnographic fieldwork, I received photos and videos taken by workers of the unclean rooms. For a number of them, the cleanliness of the dormitory is related to the design of the room and the wings, with the double bed system contributing to challenges with cleanliness. Without adequate space to put up their luggage and clothes, participants suggest that clothes and laundry are often left lying around. While walking through his room with a mobile camera, noted a participant, "How can the room be clean? How can the workers keep things clean when we are staying like this, you tell me." The survey further points to this concern about the unclean dormitory conditions. To the statement "The room where I am staying is unhygienic," 13.1% participants indicated they "somewhat agreed," 27.3% "agreed," and 31.3% "strongly agreed" (see **Figure 3**).

Lack of Toilet Facilities

The participants in our research consistently note the absence of adequate toilet facilities in the dorms. Participants often pointed out that for a block of five rooms, with 20 workers in a room, there are five toilets and five shower spaces. These infrastructures are not adequate as there are often long queues, and the facilities remain unclean. One participant noted, "Toilet and shower facility is not enough, and there is always a long line. This is the problem in the morning. I have to wake up very early at 4 a.m. to use the toilet, and then I am tired the

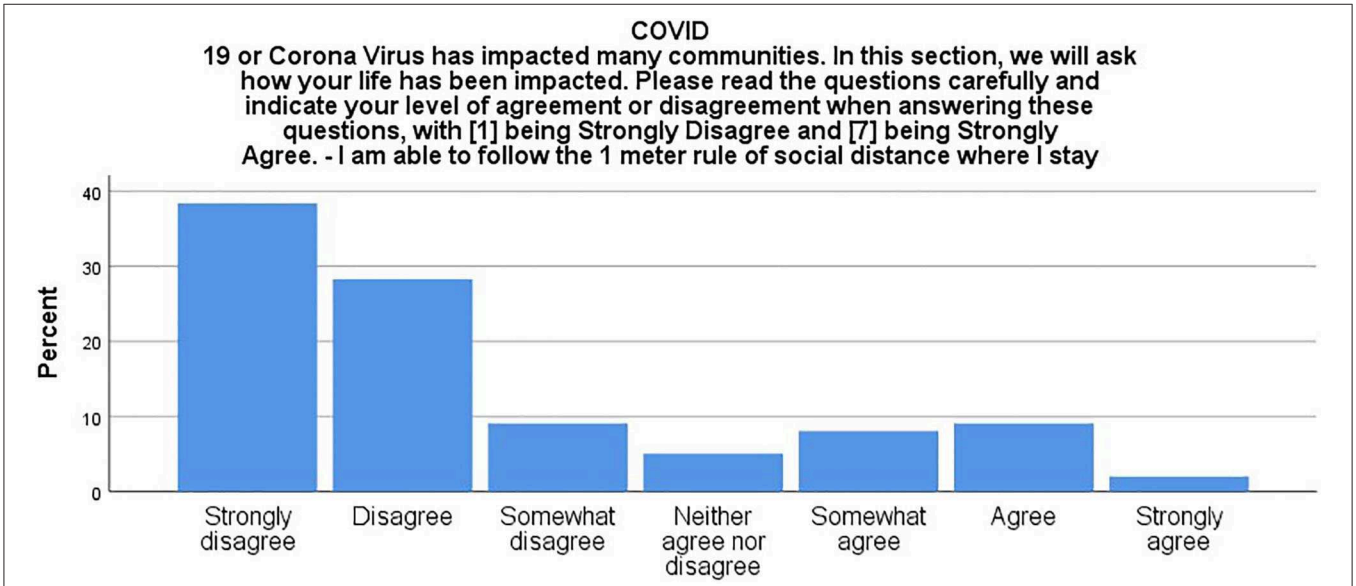


FIGURE 1 | Distribution of participants reporting their ability to follow one meter social distance rule.

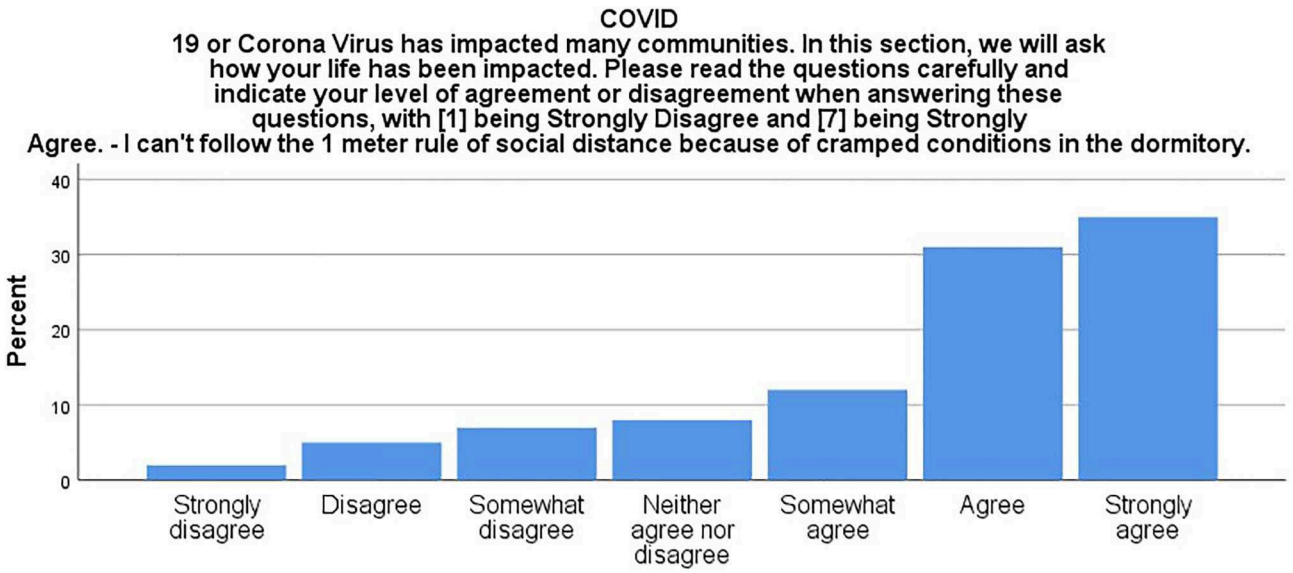


FIGURE 2 | Distribution of participants reporting inability to follow the one meter rule because of cramped conditions.

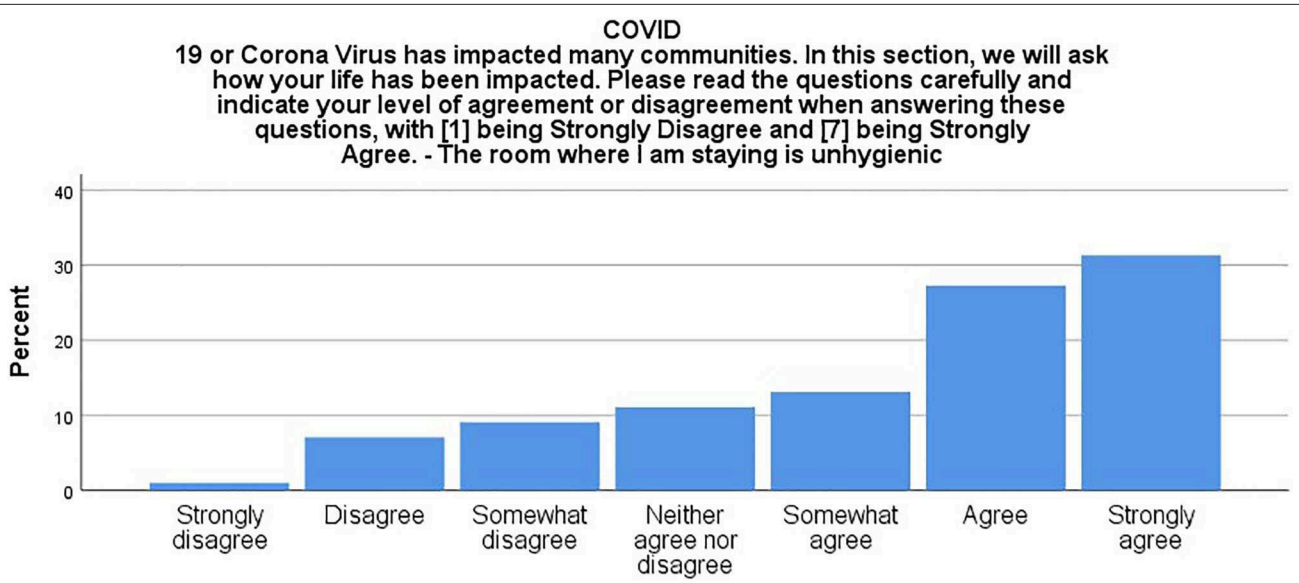


FIGURE 3 | Distribution of participants reporting their rooms as unhygienic.

whole day." Not having enough toilet translates into difficulties at the workplace, including difficulties in following workplace instructions and accidents at the workplace. This is noted by a participant, "How can a worker do the work in the site when he is tired because he wakes up very early in the morning to use the toilet?" This shortage of toilet facilities at places of accommodation is further exacerbated often by the lack of adequate toilet facilities and water at the workplace. Amidst COVID-19, participants express their anxiety about the toilets not being cleaned adequately amidst COVID-19. In the survey, in response to the statement, "The toilet where I am staying is dirty," 10.1% respondents "somewhat agree," 25.3% respondents "agree" and 30.3% respondents "strongly agree" (see **Figure 4**).

Lack of Soap and Water

Participants in the interviews shared that they are unable to wash their hands with soap regularly because of the limited supplies of soap and water. Noted a participant, "There is often not enough water after everyone uses water." In the articulations of another participant, "There is no soap. I have not received my salary yet. So I can't buy soap. The brothers (referring to NGO workers) came once and they have not returned yet." When asked about

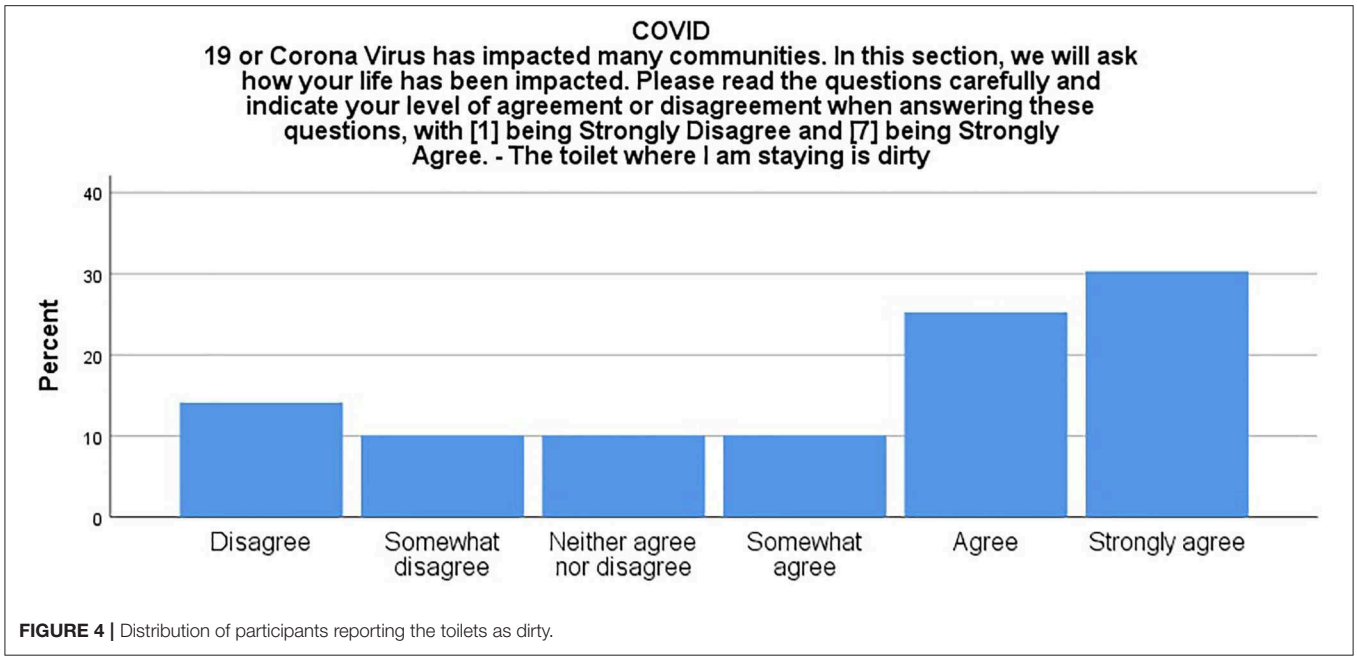


FIGURE 4 | Distribution of participants reporting the toilets as dirty.

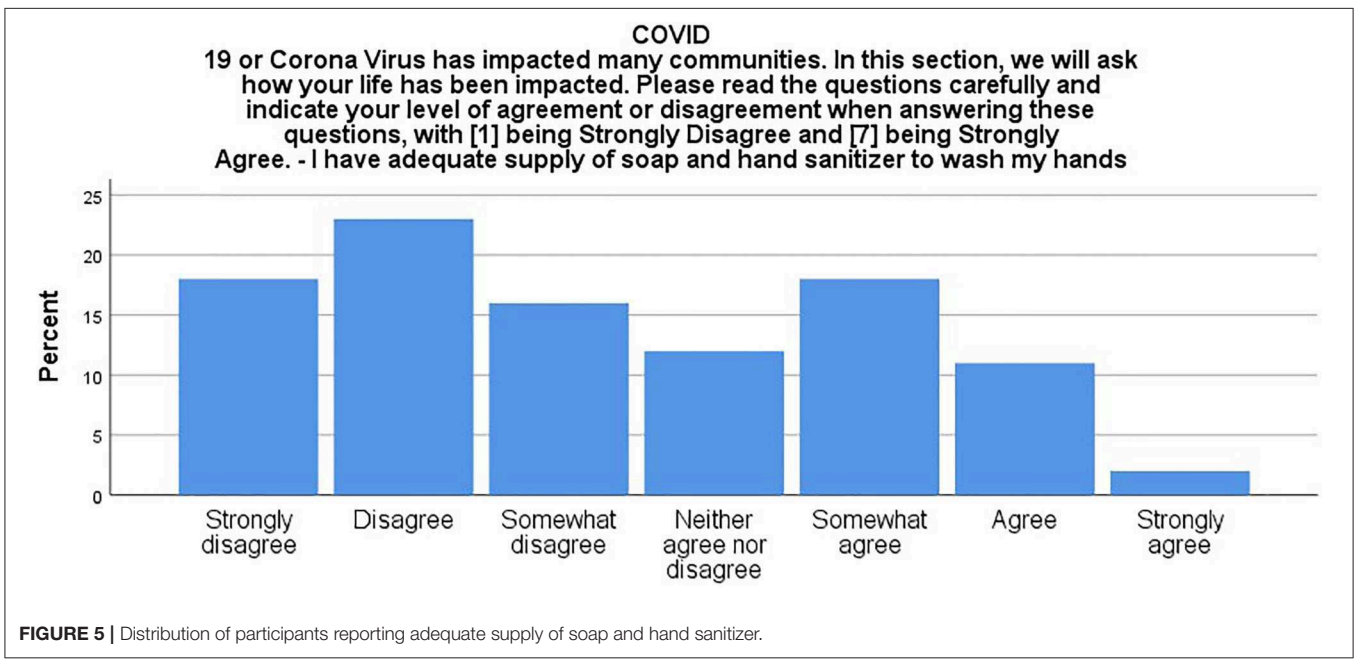


FIGURE 5 | Distribution of participants reporting adequate supply of soap and hand sanitizer.

the availability of soap and sanitizer in the survey, 18% of the participants indicated that they "strongly disagreed" with the statement, "I have adequate supply of soap and hand sanitizer to wash my hands." Moreover, 23% stated that they "disagreed," 16% "somewhat disagreed," 12% "neither agreed nor disagreed," 18% "somewhat agreed," 11% "agreed," and 2% "strongly agreed" (see **Figure 5**).

Similarly, 31.6% of the participants indicated that they "strongly disagreed" with the statement "I have adequate supply of water to wash my hands." Moreover, 22.4% stated that they "disagreed," 10.2% "somewhat disagreed," 7.1% "neither agreed nor disagreed," 14.3% "somewhat agreed," 12.2% "agreed," and 2% "strongly agreed" (see **Figure 6**).

Food Insecurity

The participants in our research have consistently noted the absence of adequate and quality food. Our advisory groups have often highlighted the lack of quality food since 2012, guiding a research study that was undertaken by CARE to examine the experiences of food insecurity among low-wage male migrant workers in Singapore, and resulting in the "Respect our food rights" campaign (Dutta, 2017a,b). For low-wage

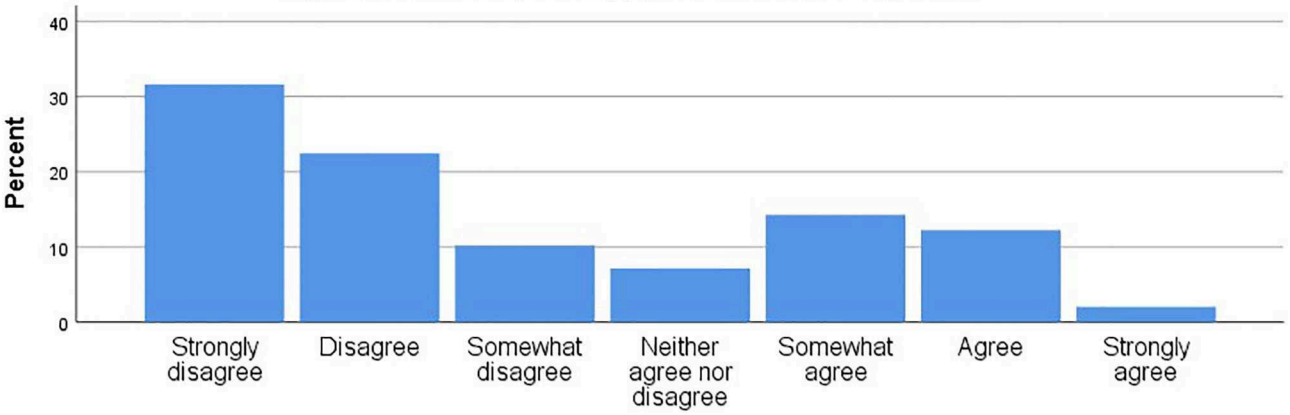


FIGURE 6 | Distribution of participants reporting adequate supply of water to wash hands.

migrant workers (predominantly Bangladeshi workers that we have worked with), the lack of access to decent food is an everyday reality of life, emergent in worker narratives as a fundamental challenge to worker health and well-being. Workers struggle with the quality of food, often reporting that they are provided with low-quality food. This is exacerbated in the midst of COVID-19. During the lock-down, workers, including those who usually cooked their own food (majority of the workers we have interviewed between 2012 and 2018 noted that they had to rely on catered food of poor quality), were supplied food by caterers. The participants noted food is often stale, has been spoilt, and is of poor quality. They pointed out that food is often oily, with poor nutrition quality.

One participant noted, "The food in the middle of COVID-19 is really bad. How can I have a strong immune system to fight the virus if this is the food I am given." In the voice of another participant, "The food is not what we eat. It is sour, and gives me heartburn. I have been sick with stomach upset two times already in the middle of the pandemic." For a number of participants, throwing away or skip a meal was the way to avoid falling sick because of the poor quality of food, "I don't even eat the catering food. The breakfast food is really bad, very sour, and it makes me sick. So usually during the whole day, I will not have eaten anything. How can I survive like this I don't know. This makes me very sad, and I have been spending days going hungry."

In the accounts offered by participants, the price of catered food had gone up amidst the pandemic, with workers having to pay larger sums of money from their meager wages. Here's a depiction of the rise in the amount of deduction for catered food, "only eating catering. Also very poor food. Deduct per month $140, which is $20 more also, already can said cruelty." For this participant and many others, the rise in the amount of money deducted for the food catering amidst the pandemic was juxtaposed in the backdrop of uncertainty about the payment of wage. This rise in the deduction amount for catering food amidst COVID-19 was reiterated by a number of participants. Moreover, our advisory group members note that in spite of the media attention to food and the stories about improvement in the quality of food, they are continuing to be served poor quality food. Noted a participant, "You see on the government websites, the minister says that the food is now improved. But please ask a worker. What will a worker say if he is not scared? I am sending you some pictures (he send me images of the poor quality food over WhatsApp). This is how the food is. How can a worker survive on this in the middle of COVID-19?" Juxtapose these accounts offered by the low-wage migrant workers in the backdrop of the recommendations for healthy eating made by the Health Promotion Board (HPB) in the midst of the pandemic[5]. The structural contexts of poor food served by caterers disrupts the individualistic behavior change recommendations for healthy practices amidst COVID-19 put forth by the state.

Erasure of Voice

"Where to even go to talk about these issues? Who will listen to us?" notes one participant. Amidst the food challenges of low-wage migrant construction workers brought up by activists, social media in Singapore has emerged as a site of conversations on the working and living conditions of low-wage migrant workers. Referring to these conversations, voices a participant, "I just want decent food. Give me my dignity. There are so

[5]The Health Promotion Board recommendations for coping with COVID-19, folded into the "Stay well" campaign, under the umbrella of the SG clean and SG United campaigns, encourage the target audience to eat healthy while in the lockdown (*https://www.healthhub.sg/programmes/170/StayWell).* The recommendations reflect the World Health Organization (WHO) recommendations for healthy eating during the lockdown (*https://www.who.int/campaigns/connecting-the-world-to-combat-coronavirus/healthyathome/healthyathome—healthy-diet*).

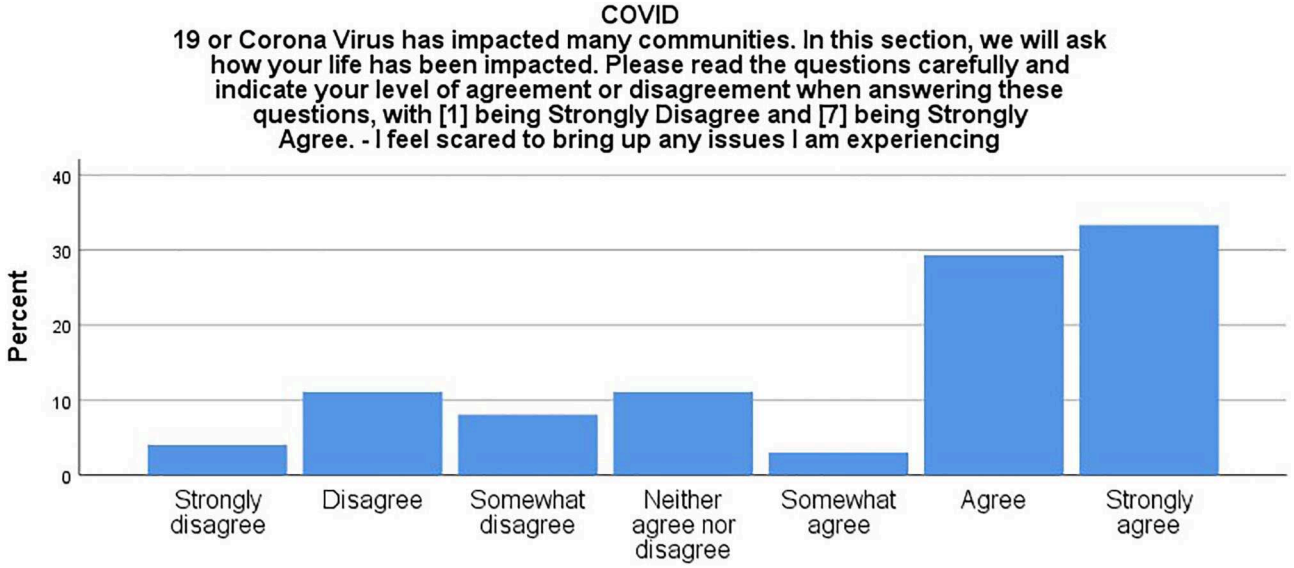


FIGURE 7 | Distribution of participants reporting fear in bringing up issues.

many Singaporeans that say on Facebook that workers shouldn't complain because we are lucky to be here." Shares another participant, "I can't really talk about any of these problems. If my boss comes to know, he will take me out of the job and send me back." Yet another participant shares, "I am scared to say anything. Saying anything will get me into trouble. My work pass will be taken away." The erasure of spaces for voicing their everyday challenges to health and well-being is situated amidst the tremendous power differentials that constitute low-wage migrant labor in Singapore.

In the backdrop of the strong structural barriers and the lack of certainty regarding the payment of wages/salaries, 33.3% of the participants indicated that they "strongly agreed" with the statement "I feel scared to bring up any issues I am experiencing." Moreover, 29.3% stated that they "agreed," 3% "somewhat agreed," 11.1% "neither agreed nor disagreed," 8.1% "somewhat disagreed," 11.1% "disagreed," and 4% "strongly disagreed" (see **Figure 7**).

Participants reported their knowledge of how to approach the Ministry of Manpower to share any challenges they face. In response to the statement, "I know how to approach the Ministry of Manpower to share any challenges I am facing," 19% "strongly disagreed," 28% "disagreed," and 19% "somewhat disagreed" while 14% "somewhat agreed" (see **Figure 8**).

In response to the statement, "If I have difficulties, I know whom to talk to" 29% "strongly disagreed," 31% "disagreed," and 10% "somewhat disagreed" while 9% "somewhat agreed" and 9% "agreed" (see **Figure 9**).

Mental Health

The participants share ongoing challenges to mental health and well-being, constituted by anxieties related to contracting COVID-19, anxieties about the inability to practice social distancing because of the crowded living arrangements and unclean toilets, anxieties about being fired or deported if they were to speak out, anxieties related to payment, and worries about their families back home. Centered in our conversations is the ongoing worry about not being able to support their families back home financially as well as a deep sense of fear over their future. For many participants, past and ongoing challenges with getting paid their salary constitute the sense of sadness they express, tied to their identities and roles as providers for their families. A participant expresses this: "I am here with all of this pain so my family can be taken care of. When my family is struggling because I can't take care of them, how can I be at peace?" This ongoing worry about the payment of salary is situated in the backdrop of state assurances of payment. One participant shares: "I don't trust anyone. I don't trust any Facebook video [referring to a message from the Minister of Manpower]." Another participant shared that he had been complaining about the non-payment of wages, and this resulted in him being fired by his employer amidst the lockdown. He shares, "the employer sent the termination letter. I was making posts on Facebook about the salary not being paid. Even if a worker speaks of what is rightfully his [referring to the salary], he can get fired." Not having a voice, the overarching sense of anxiety, and various forms of disciplining the workers are subjected to result in the everyday challenges to mental health amidst COVID-19. Shares a participant, "I am scared. I worry I will not be able to see my family. I am scared I will not be able to send money home. Last month, I did not get the salary. Now, I am worried what will happen if I am infected." Many workers in the digital spaces (such as Facebook groups run by workers) shared their worries about the non-payment of salary. In spite of the state's assurance about payment, workers shared that they worried whether the payment would actually reach them. This sense of worry about the payment of the salary is evident throughout the interviews. Noted a participant, "I haven't been able to send money home for 2 months. My family back

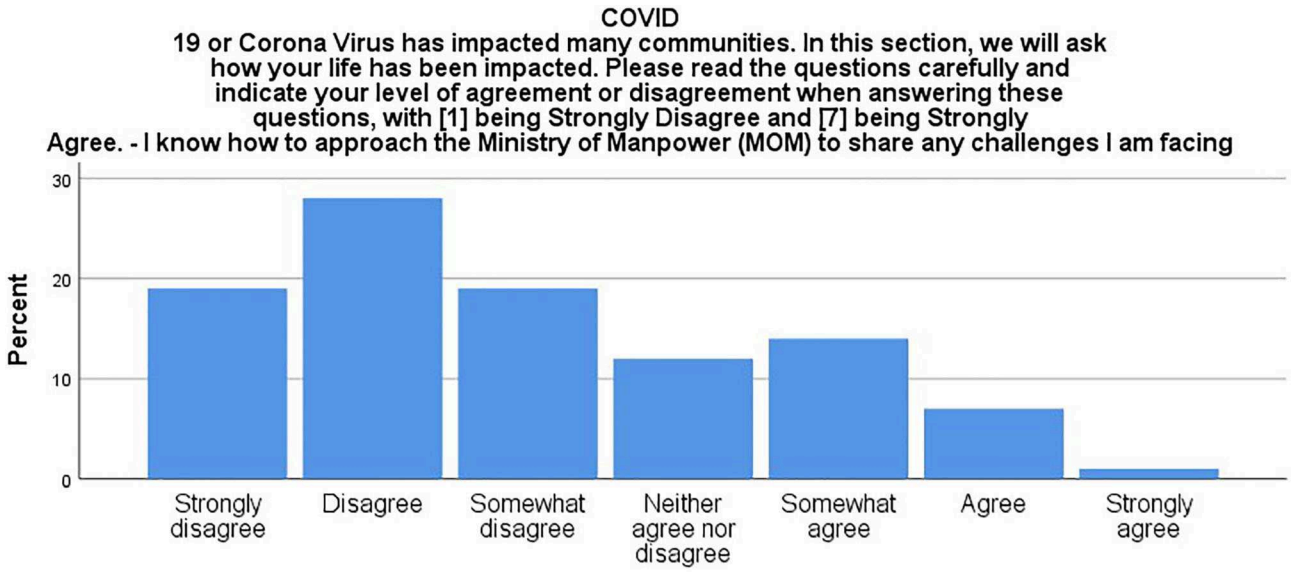


FIGURE 8 | Distribution of participants reporting knowledge of how to approach the MOM.

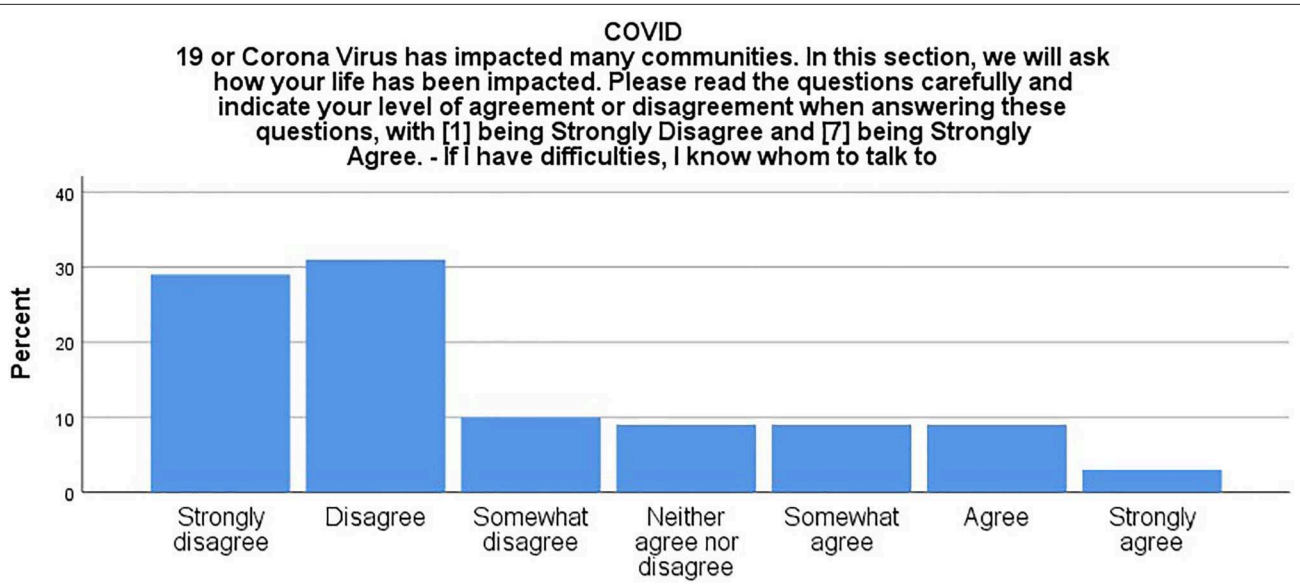


FIGURE 9 | Distribution of participants reporting knowledge of whom to talk to when experiencing difficulties.

home is really struggling. My mother said to me the other day when I spoke with her, she doesn't know what she will feed my family. I think about this and cry."

In a Facebook group created and hosted by low-wage migrant workers, anxieties about infections are expressed amidst the fear of being separated from families at home. Many posts refer to the prison-like conditions of the dorm rooms. In a post, a participant points out that he feels depressed, being imprisoned with 15 other workers in a room and worried about the likelihood of infection because of the cramped condition in the rooms.

Another participant voices, "This room where I am staying, I only see the walls and stay inside. I don't know what is happening around me. We are moved from room to room and new people keep coming. I don't know what is happening in the dormitory and why the movement of the workers. This makes me worry." Not having access to information about the steps being taken by the dormitory and the reasons behind the movement of workers from room to room emerge as challenges to mental health. Notes a participant, "I am scared. This corona virus, one brother in the room was infected. So I am scared. No one really explains anything. A few of us have been moved from one room to another. I am very scared." This feeling of being scared is tied to the feeling of sadness many participants express. One participant shares: "I cry whenever I see my wife on the mobile. I don't

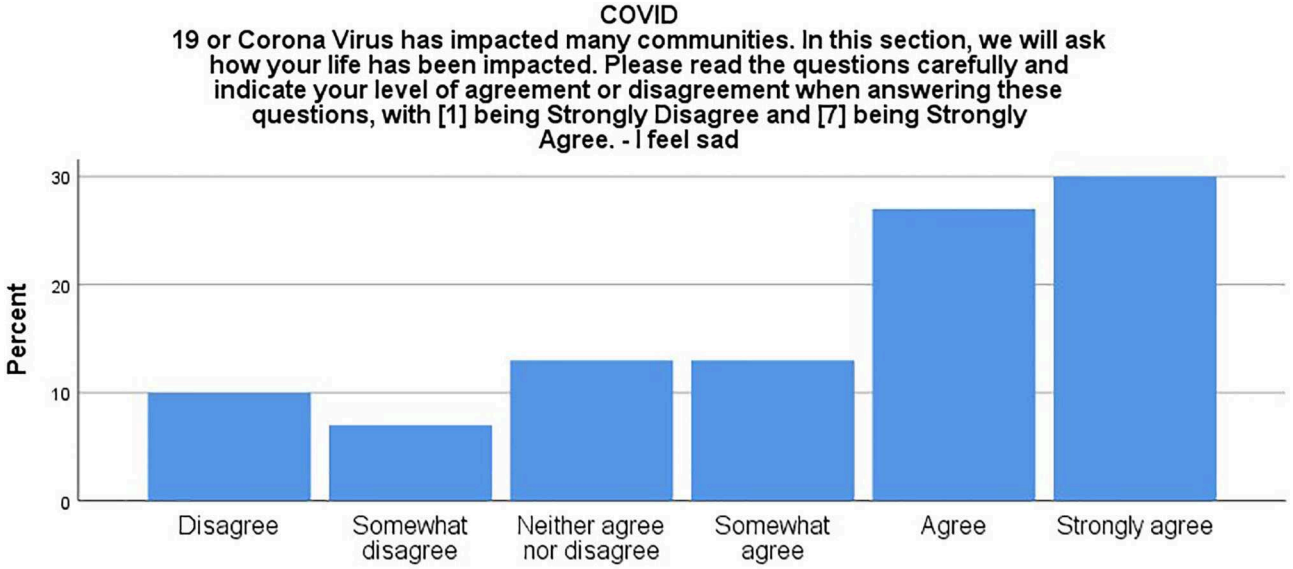


FIGURE 10 | Distribution of participants reporting feeling sad.

know if I will see the children again, what will happen. With so many people (referring to the room), I am scared I will become infected. Worrying about it, I sometimes cry." In response to the statement, "I feel sad," 13% "somewhat agreed," 27% "agreed," and 30% "strongly agreed" (see **Figure 10**).

In response to the statement, "I feel depressed," 11% "somewhat agreed," 28% "agreed," and 29% "strongly agreed" (see **Figure 11**).

Juxtapose these articulations of structurally constituted everyday experiences of health in the backdrop of the state's SG United campaign with recommendations of individual behaviors to upkeep mental health and well-being[6]. The individualized, behaviorally directed narrative of self-help propelled by the state is ruptured through the accounts of structural and communicative violence expressed by the low-wage migrant workers.

DISCUSSION

This article, anchored in the CCA attends to the ongoing erasure of low-wage migrant workers in Singapore, amidst the active work of depoliticizing the exploitation of low-wage migrant workers and propping up the individualizing ideology of altruism. The meanings of health co-constructed by the low-wage migrant workers amidst the COVID-19 outbreak in dormitories housing them foregrounds the interplays of culture, structure, and agency, juxtaposed in the backdrop of the hegemonic ideology of service delivery that constitutes the state-civil society nexus. Activists pointing to the communicative erasures of earlier accounts documenting the poor housing and food conditions have been attacked in public and digital media discourses, supported by hegemonic structures (see for instance the targeted attack on the activist Kokila Annamalai)[7]. Salient amid the COVID-19 outbreaks in the dormitories, the instrument of Prevention of Online Falsehoods and Manipulation Act (POFMA) created by the state to supposedly regulate "fake news" has been rhetorically mobilized[8]. This then serves as the backdrop of attacks on activists pointing to ongoing challenges with food experienced by low-wage migrant workers. Similarly, civil society organizations that have publicly interrogated state policies related to migration face a wide range of challenges amidst their COVID-19 responses. The authoritarian impulse of extreme neoliberalism seeds and circulates the rhetoric of the "anti-national" to suppress dissenting voices, thus legitimizing techniques of disciplining as necessary responses of good governance (Bruff and Tansel, 2019). Extreme neoliberalism sustains the free market ideology as an organizing framework even as its narrative has been thoroughly disrupted by the everyday empirical accounts of those struggling with its violent effects at the margins. As an extreme form of governmentality, it performs layers of communicative inversions amid techniques of disciplining and deployment of "communicative inversions"

[6]The "Stay well" campaign discussed earlier encourages the target audience to stay positive, suggesting guided imagery, mindfulness, progressive relaxation, and deep breathing (*https://www.healthhub.sg/programmes/170/StayWell).*

[7]In the opinion piece published in The Online Citizen, the Singapore human rights activist Jolovan Wham outlines the ideology of authoritarian repression that targets activists, *https://www.onlinecitizenasia.com/2020/05/04/advocacy-activist-harassment-and-solidarity/?fbclid$=$IwAR2-lHw-8z3pKy8ffHKaoVeWuc1KjdH2K6_t-IBa25n6yAvJYG6awR0le8s.*

[8]POFMA directive has been ordered to activists for making claims about worker payments (see *https://www.channelnewsasia.com/news/singapore/foreign-workers-dorms-pofma-alex-tan-singapore-states-times-12614908*); POFMA has been referred to by the Minister of Home Affairs in relationship to the images of poor food circulating on social media (*https://www.straitstimes.com/singapore/some-people-spreading-fake-news-about-foreign-worker-dorms-to-incite-violence-shanmugam*).

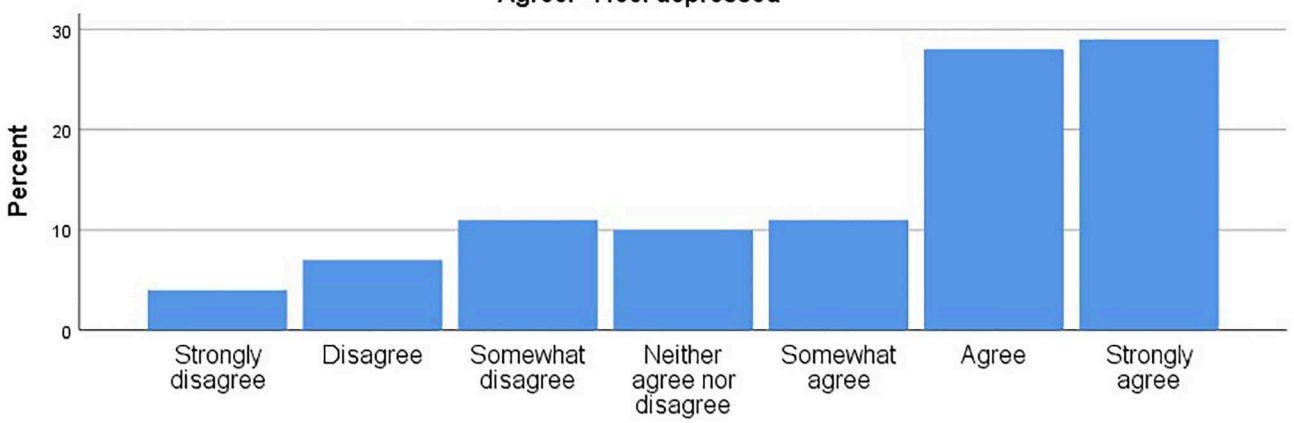


FIGURE 11 | Distribution of participants reporting feeling depressed.

through state-controlled media infrastructures to project its failures as excellence in governmentality.

Constituted amidst these strategies of calibrating authoritarian control are the experience of communicative violence expressed by the low-wage migrant workers in Singapore, not knowing whom to go to raise their complaints, frightened to raise complaints because of the precarity of their work, and often living amidst structurally constituted communicative gaps. These forms of communicative violence are situated amidst structural violence that is magnified by the trajectories of COVID-19, and that forms the hegemonic organizing of "unfree labor" in Singapore (see Yea and Chok, 2018). Communicative inequality, inequality in distribution of information and voice infrastructures, is intricately tied to the material inequalities experienced by low-wage migrant workers (Dutta, 2008). The violence of extreme neoliberalism is worked into the various forms of disciplining that work actively to silence the voices of workers (Springer, 2012, 2015). The various forms of disciplining the workers experience, from being surveilled to being terminated for speaking up work together to perpetuate a fundamental form of violence ingrained in fear.

The health of low-wage migrant workers amidst the COVID-19 outbreaks attends to the interplays of authoritarian labor management and policy implementation failures that constitute the extreme neoliberal policies of Singapore. Paradoxically, the challenges to the health and well-being of low-wage migrant workers in Singapore exists alongside the claims to technocratic efficient management of the pandemic through tools of contact tracing and quarantining celebrated by global organizations such as the WHO and the World Economic Forum (WEF)[9]. The very technologies of authoritarian control that work on one hand in legitimizing efficient pandemic management while upholding the neoliberal ideology of "open borders" also have historically rendered invisible the structural violence within which low-wage migrant workers negotiate their lives through disciplining and control. The pandemic outbreak in low-wage migrant worker dormitories makes visible these poor conditions of health, work, and well-being (Dutta, 2020a,b). What this article demonstrates is that the everyday threats to health and well-being that are routinized into the routine management of low-wage migrant workers in Singapore are exacerbated by the pandemic. Inherent in the state's official narrative that articulates the impossibility of anticipating the outbreak is its systematic efforts of individualizing behavior and unseeing low-wage migrant workers in discursive spaces[10]. Responding to the outbreaks in the dormitories housing low-wage migrant workers, the state's reporting framework separates the migrant worker infections from the general population (Han, 2020). This framework of differentiation underlies a racist ideology that is also reflected in the state's spatial management of low-wage migrant workers, placing them on the invisible outskirts of the "smart city," marking their bodies as targets of neoliberal management regimes, and targeting them through techniques of disciplining[11].

[9]An entire infrastructure of think tanks, pundits, experts, and journalists are mobilized economically and politically to circulate the "Singapore model" as a miracle, as an exemplar of "exceptional political leadership." Consider for instance, the ongoing work performed by the journalist Fareed Zakaria in holding up Singapore as a model of efficient governance driven by exceptional leadership (*https://www.youtube.com/watch?v=hVUEoFyokqg*).

[10]Consider the claim that the outbreaks are connected to communal living of migrant workers, suggesting cultural-behavioral roots of the infections (*https://www.cnbc.com/video/2020/05/06/coronavirus-singapore-minister-on-migrant-dormitories-during-outbreak.html*).

[11]In the backdrop of what is termed by the official state narrative as the "Little India riot" (Kaur, Tan, and Dutta, 2016), various technologies of surveillance and disciplining have been put in place in Little India, the space where large numbers of low-wage migrant workers tend to gather on Sundays, their weekly day-off. Large structured of flood lights have taken over Little India, with streets filled with auxiliary and police forces to observe, discipline, and control the workers.

Amidst their anxieties about the structural constraints that limit their ability to practice preventive behaviors, the low-wage migrant workers who participated in this study narrate accounts of everyday erasure. This erasure is constituted amid strict laws that prevent migrant worker organizing and silence the voices of migrant workers, the power over work and temporary visas held by employers, and the lack of structures of articulation. A political economy of mediation fosters communicative inequality (Dutta, 2016), with migrant workers living in fear amidst the temporariness of their migrant status and without the access to infrastructures for voicing their everyday challenges with poor working conditions, poor housing, poor food, and poor transportation. These structural features of work and livelihood constitute the crowded living conditions that shape the spread of COVID-19 among low-wage migrant workers in Singapore. The structural violence experienced by low-wage migrant workers in Singapore is situated amidst policies of surveillance and policing that mark migrant workers to be controlled. Paradoxically then, even as structural resources for health and well-being have been largely absent, various technologies of surveillance and containment have been put into place targeting the low-wage migrant worker. This coupling of technologies of surveillance and the absence of the fundamental structures of health depicts a racist-neoliberal ideology that sees the low-wage migrant worker as a disposable body in the circuits of accumulation of primitive capital. The specific context of extreme neoliberalism in Singapore and its failures further renders visible the failure of the behavior change paradigm of health communication that systematically erases structures while simultaneously putting forth recommendations for individual behavior change such as staying home, maintaining physical distance, washing hands, and wearing masks. The zeitgeist of the neoliberal behavior change framework, working to promote individualized responses that simultaneously erase questions of structural transformation, is confronted with its failures in Singapore and elsewhere across the globe. This suggests the urgency of radically transforming health communication, moving beyond its occasional lip service to structures under the hegemonic narrative of addressing health disparities while keeping the neoliberal structures intact, to revolutionary politics co-constructed in solidarity with the working classes locally, nationally, and globally, urgently building communicative registers for working class solidarity in disrupting and dismantling neoliberalism, and simultaneously building socialist political economies of health and well-being.

The essay wraps up by theorizing the work of co-creating communicative equality by building democratic infrastructures for migrant voices, which emerge as vital resources in addressing the pandemic, and offer the registers for post-pandemic transformations of politics, economics, and social organizing. Some of the key findings that are presented in this manuscript were earlier presented in the form of white papers designed to generate conversations in Singapore, having contributed to media advocacy driven by the advisory group (Dutta, 2020a,b). Drawing on the key tenets of the CCA, these conversations, driven by low-way migrant workers, sought to create registers for structural transformation. The erasure of voice, the absence of information infrastructures, and the absence of infrastructures for voice constitute the context of hyper-precarity of migrant work. The "interplay of neoliberal labor markets and highly restrictive immigration regimes" (Lewis et al., 2015) that depict hyper-precarity of low-wage migrant work in Singapore is rooted in communicative violence, the strategic erasure of communicative infrastructures for migrant worker voices. This communicative violence forms the "smart city" infrastructure of Singapore, deploying a wide array of strategies of erasure to render invisible the pain and poor health experienced by low-wage migrant workers. The erasure of the voices of low-wage migrant workers is a vital tool in legitimizing. Singapore's extreme neoliberalism, upholding its narrative of smart governance through projections of technology, participation, and sustainability. Communicative violence is a salient organizing feature of extreme neoliberalism. This communicative violence materializes in an overarching sense of fear and feelings of depression expressed by the workers, situated amidst fear of facing consequences of losing job or being deported if they speak up about the challenges being experienced (as evidenced in the findings, workers offer accounts of losing jobs for speaking up/out). Beyond the health challenges of COVID-19 infection, the participant observations, interviews and survey point to the challenges with mental health experienced by the low-wage migrant workers. These conditions of poor mental health make visible a vital site of health negotiation at the margins of extreme neoliberal societies amidst the pandemic.

Finally, the narratives offered by low-wage migrant workers foreground the structural violence that constitutes the infrastructure of preventive behaviors related to COVID-19. The everyday practices of recommended behaviors such as maintaining 1-meter physical distance and regularly washing hands with soap and water are situated amidst the infrastructures of poor housing, poor sanitation, limited supply of water, and lack of cleaning resources.

Moreover, the behavioral recommendation mandating the workers to stay in their rooms to prevent the spread of COVID-19 constitutes the paradox of over-crowded rooms that are rife with opportunities for the virus to transmit. Contrast the reality of poor, over-crowded housing with the cultural-behavioral hegemonic accounts of workers crowding in groups. The structural violence of poor housing, poor sanitation, and poor food that forms the everyday context of health of low-wage migrant workers is exacerbated by the pandemic, magnifying the effects on worker health and well-being. What the COVID-19 outbreak in migrant worker dormitories makes visible is the limit of the "Singapore model" as an exemplar of extreme neoliberalism in public health response, with gross inequalities normalized into its health and care infrastructures. The voices of the participants point to the lack of access to healthcare in the everyday contexts of health, the card-based security system used at the gates, and the lack of adequate conditions of housing as the reasons for the accelerated spread of the pandemic among low-wage migrant workers. The recognition of deeply unequal structures as the sites of the epidemic offer transformative registers for justice, communicative equality, and

worker organizing, centering care as an organizing narrative for structural transformation. Note here that amidst the extensive global media coverage that documented the failures of Singapore's COVID-19 response and highlighted the poor treatment of low-wage migrant workers, the state responded with policy proposals for improving the housing conditions for the workers. However, any such response, in the absence of communicative equality and communicative justice, is episodic, performative, and not held accountable. In the absence of infrastructures for democracy, there are no existing democratic mechanisms to hold the ruling People's Action Party (PAP) accountable or actually measuring its effectiveness in delivering the policy proposals (Dutta, 2018b; Thompson, 2019). This is the inherent paradox of extreme neoliberalism, that its seductions of accounting to prop up the neoliberal structure exist alongside the absolute lack of infrastructures of accountability that exist outside of the purviews of the hegemonic PAP. Moreover, the authoritarian techniques of erasure that already erase voices of dissent perform violent erasures of migrant worker voices, who are left without access to structures of claims-making into the state. As reflected in the pandemic electoral conversations, the plight of the low-wage migrant workers which constituted the fundamental failure of the neoliberal state, are once again entirely erased from the conversation, missing from the articulations and debates. Even the opposition parties largely erase the everyday struggles and plight of low-wage migrant workers from the discursive space (there are some exceptions, such as the Singapore Democratic Party's platform that calls for a minimum wage framework for all workers and the SDP politician-public health expert Paul Ananth Tambyah who has highlighted consistently the public health failures in addressing the health of low-wage migrant workers). Culture-centered advocacy imagines an actual politics of resistance, fundamentally suggesting strategies for dismantling extreme neoliberalism by foregrounding an ethic of voice, solidarity and justice rooted in communicative equality. The ability to craft alliances between the essential rights of Singaporeans workers and migrant workers lies at the heart of building socially just futures (Dutta and Zapata, 2018; Falnikar et al., 2019).

Given the sense of anxiety expressed by the participants, this manuscript does not disclose the locations of the various living arrangements or compare the lived experiences of workers across the different forms of arrangements to protect the confidentiality of the participants. Also, the manuscript specifically reports on the key emergent themes that were of salience to the advisory group, suggesting that other issues related to the pandemic are not included here. For instance, although mask wearing is a key part of the pandemic response and did appear in the interviews, the advisory group noted that they largely had access to masks in the dormitories as well as at workplaces[12].

[12]At the time of conducting the interviews, various organized campaigns were being carried out to distribute masks to low-wage male migrant workers as well as foreign domestic workers. For instance, the community-grounded campaign, MaskForce, was organizing fundraising to donate masks. Several other community-grounded activities had started for organizing masks for the workers.

The excerpts from the interviews are truncated to protect the identity of the participants. Given the snowball method of recruitment for the survey by circulating the survey link in the second phase of the study, quality control is difficult. However, a large majority of the data gathering took place over the phone after making initial contact over Facebook messenger, Telegram, or WhatsApp, enabling verification. When the analysis was run with the phone-only sample, the same patterns were retained for the reported variables. Also, the current report of phase two of the study is based on a relatively small sample size. However, the triangulation of the survey data with the in-depth interviews and participant observations strengthens the validity of the study. This study demonstrates the robust data gathering infrastructures of the CCA in place amidst a crisis, anchored in an already existing sustained academic-community partnership in the form of an engaged advisory group of community members, advocates, and researchers involved on an ongoing basis in developing research frameworks and solutions for addressing the challenges to migrant worker health. As noted earlier, the emergent findings formed the basis of two white papers that served as registers for health advocacy amidst COVID-19 (Dutta, 2020a,b), leading to widespread local and international media coverage attending to the failures of the Singapore pandemic-response framework. Also, given the challenges with catered food voiced by the workers amidst the pandemic, the existing digital campaign infrastructure of "Respect our Rights" was utilized to disseminate already existing campaign messages on the quality of food, putting forth specific infrastructure-based demands that have been voiced by the workers on an ongoing basis (https://www.facebook.com/Migrant-Workers-Rights-SG-1557463061204402/). The CCA offers a register for both theoretically anchoring migrant health in transformative imaginaries and in co-creating an actual politics of structural transformation at the margins by seeding communicative infrastructures for voices of the margins.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because confidentiality of the participants need to be maintained. Requests to access the datasets should be directed to m.j.dutta@massey.ac.nz.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Low Risk Human Ethics, Massey University. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

MD has designed the study, conducted the fieldwork, analyzed the data, and wrote this report.

REFERENCES

Baey, G., and Yeoh, B. S. (2018). "The lottery of my life": migration trajectories and the production of precarity among Bangladeshi migrant workers in Singapore's construction industry. *Asian Pac. Migr. J.* 27, 249–272. doi: 10.1177/0117196818780087

Baey, G., and Yeoh, B. S. A. (2015). "Migration and precarious work: negotiating debt, employment, and livelihood strategies amongst Bangladeshi migrant men working in Singapore's construction industry," in *Migrating out of Poverty Working Paper 26* (Brighton: University of Sussex). Available online at: http://migratingoutofpoverty.dfid.~gov.uk/documents/wp26-baey-yeoh-2015-migration-and-precarious-work.pdf (accessed May 1, 2020).

Bal, C. S. (2015). Production politics and migrant labour advocacy in Singapore. *J. Contemp. Asia.* 45, 219–242. doi: 10.1080/00472336.2014.960880

Bates, B. R., Marvel, D. L., Nieto-Sanchez, C., and Grijalva, M. J. (2019). Painting a community-based definition of health: a culture-centered approach to listening to rural voice in Chaquizhca, Ecuador. *Front. Commun.* 4:37. doi: 10.3389/fcomm.2019.00037

Bruff, I. (2014). The rise of authoritarian neoliberalism. *Rethink. Marx.* 26, 113–129. doi: 10.1080/08935696.2013.843250

Bruff, I., and Tansel, C. B. (2019). Authoritarian neoliberalism: trajectories of knowledge production and praxis. *Globalizations* 16, 233–244. doi: 10.1080/14747731.2018.1502497

Dutta, M., Sastry, S., Dillard, S., Kumar, R., Anaele, A., Collins, W., and Spinetta, C. (2017). Narratives of stress in health meanings of African Americans in Lake County, Indiana. *Health Commun.* 32, 1241–1251. doi: 10.1080/10410236.2016.1204583

Dutta, M. J. (2004a). Poverty, structural barriers, and health: a Santali narrative of health communication. *Qual. Health Res.* 14, 1107–1122. doi: 10.1177/1049732304267763

Dutta, M. J. (2004b). The unheard voices of Santalis: communicating about health from the margins of India. *Commun. Theory.* 14, 237–263. doi: 10.1111/j.1468-2885.2004.tb00313.x

Dutta, M. J. (2007). Communicating about culture and health: theorizing culture-centered and cultural sensitivity approaches. *Commun. Theory* 17, 304–328. doi: 10.1111/j.1468-2885.2007.00297.x

Dutta, M. J. (2008). *Communicating Health: A Culture-Centered Approach*. London: Polity.

Dutta, M. J. (2015). Decolonizing communication for social change: a culture-centered approach. *Commun. Theory.* 25, 123–143. doi: 10.1111/comt.12067

Dutta, M. J. (2016). *Neoliberal Health Organizing: Communication, Meaning, and Politics, Vol. 2*. New York, NY: Routledge. doi: 10.4324/9781315423531

Dutta, M. J. (2017a). Migration and health in the construction industry: culturally centering voices of Bangladeshi workers in Singapore. *Int. J. Environ. Res. Public Health* 14:132. doi: 10.3390/ijerph14020132

Dutta, M. J. (2017b). "Negotiating health on dirty jobs: culture-centered constructions of health among migrant construction workers in Singapore," in *Culture, Migration, and Health Communication in a Global Context*, eds Y. Mao and R. Ahmed (New York, NY: Routledge), 45–59. doi: 10.4324/9781315401348-4

Dutta, M. J. (2018a). Culture-centered approach in addressing health disparities: communication infrastructures for subaltern voices. *Commun. Methods Meas.* 12, 239–259. doi: 10.1080/19312458.2018.1453057

Dutta, M. J. (2018b). Culturally centering social change communication: subaltern critiques of, resistance to, and re-imagination of development. *J. Multicul. Discourses* 13, 87–104. doi: 10.1080/17447143.2018.1446440

Dutta, M. J. (2019a). Digital transformations, smart cities, and displacements: tracing the margins of digital development. *Int. J. Media Stud.* 1, 1–21. Available online at: https://www.efluniversity.ac.in/Journals-Communication/IJMS1_Dutta.pdf

Dutta, M. J. (2019b). What is alternative modernity? Decolonizing culture as hybridity in the Asian turn. *Asia Pac. Media Educ.* 29, 178–194. doi: 10.1177/1326365X19881256

Dutta, M. J. (2020a). *Infrastructures of Housing and Food for Low-Wage Migrant Workers in Singapore*. Palmerston North: Center for Culture-centered Approach to Research and Evaluation (CARE).

Dutta, M. J. (2020b). *Structural Constraints, Voice Infrastructures, and Mental Health Among Low-Wage Migrant Workers in Singapore: Solutions for Addressing COVID19*. Palmerston North: Center for Culture-centered Approach to Research and Evaluation (CARE).

Dutta, M. J., and Basu, A. (2008). Meanings of health: interrogating structure and culture. *Health Commun.* 23, 560–572. doi: 10.1080/10410230802465266

Dutta, M. J., and Jamil, R. (2013). Health at the margins of migration: culture-centered co-constructions among Bangladeshi immigrants. *Health commun.* 28, 170–182. doi: 10.1080/10410236.2012.666956

Dutta, M. J., and Kaur-Gill, S. (2018). Precarities of migrant work in Singapore: migration, (im) mobility, and neoliberal governmentality. *Int. J. Commun.* 12, 4066–4084.

Dutta, M. J., Pandi, A. R., Mahtani, R., Falnikar, A., Thaker, J., Pitaloka, D., and Sun, K. (2019). Critical health communication method as embodied practice of resistance: culturally centering structural transformation through struggle for voice. *Front. Commun.* 4:67. doi: 10.3389/fcomm.2019.00067

Dutta, M. J., and Zapata, D. B. (Eds.). (2018). *Communicating for Social Change: Meaning, Power, and Resistance*. Singapore: Springer. doi: 10.1007/978-981-13-2005-7

Falnikar, A., Lyn, T. E., Ganchoudhuri, S., and Dutta, M. J. (2019). Discursive constructions of income inequality in neo-liberal Singapore. *J. Creat. Commun.* 14, 132–146. doi: 10.1177/0973258619851979

Han, K. (2020, May 20). Singapore is trying to forget migrant workers are people. *Foreign Policy*. Retrieved from https://foreignpolicy.com/2020/05/06/singapore-coronavirus-~pandemic-migrant-workers/

Jamil, R., and Kumar, R. (2020). Culture, structure, and health: narratives of low-income Bangladeshi migrant workers from the United Arab Emirates. *Health Commun.* 1–12. doi: 10.1080/10410236.2020.1750773

Juego, B. (2018). Authoritarian neoliberalism: its ideological antecedents and policy manifestations from Carl Schmitt's political economy of governance. *Adm. Cult.* 19, 105–136. doi: 10.32994/ac.v19i1.209

Kaur, S., Tan, N., and Dutta, M. J. (2016). Media, migration and politics: the coverage of the little India riot in The Straits Times in Singapore. *J. Creat. Commun.* 11, 27–43. doi: 10.1177/0973258616630214

Kong, L. (2018). *The Ideological Alignment of Smart Urbanism in Singapore. Urban Studies Journal YouTube Channel*. Available online at: https://www.youtube.com/watch?v=SVTaChMuZKs (accessed May 7, 2019).

Kong, L., and Woods, O. (2018). The ideological alignment of smart urbanism in Singapore: critical reflections on a political paradox. *Urban Stud.* 55, 679–701. doi: 10.1177/0042098017746528

Lewis, H., Dwyer, P., Hodkinson, S., and Waite, L. (2015). Hyper-precarious lives: migrants, work and forced labour in the Global North. *Prog. Hum. Geogr.* 39, 580–600. doi: 10.1177/0309132514548303

Lindquist, J., Xiang, B., and Yeoh, B. S. (2012). Opening the black box of migration: brokers, the organization of transnational mobility and the changing political economy in Asia. *Pac. Aff.* 85, 7–19. doi: 10.5509/20128517

Low, D. (2014). "Governing in the new normal," in *Hard Choices: Challenging the Singapore Consensus*, eds D. Low and S. Vadaketh (Singapore: NUS Press), 178–187. doi: 10.2307/j.ctv1ntgrm.19

Newman, S., Cheng, T., Ghahate, D. M., Bobelu, J., Sandy, P., Faber, T., and Shah, V. O. (2014). Assessing knowledge and attitudes of diabetes in Zuni Indians using a culture- centered approach. *PLoS ONE* 9:e99614. doi: 10.1371/journal.pone.0099614

Pow, C. P. (2014). License to travel: policy assemblage and the 'Singapore model'. *City* 18, 287–306. doi: 10.1080/13604813.2014.908515

Rahim, L. Z., and Barr, M. D. (eds). (2019). "Introduction: authoritarian governance in Singapore's developmental state," in *The Limits of Authoritarian Governance in Singapore's Developmental State* (Singapore: Palgrave Macmillan), 1–25. doi: 10.1007/978-981-13-1556-5_1

Sastry, S., Stephenson, M., Dillon, P., and Carter, A. (2019). A meta-theoretical systematic review of the culture-centered approach to health communication:

toward a refined, "nested" model. *Commun. Theory*. doi: 10.1093/ct/qtz024

Springer, S. (2012). Neoliberalising violence: of the exceptional and the exemplary in coalescing moments. *Area* 44, 136–143. doi: 10.1111/j.1475-4762.2012.01084.x

Springer, S. (2015). *Violent Neoliberalism: Development, Discourse, and Dispossession in Cambodia*. New York, NY: Springer. doi: 10.1057/9781137485335

Tan, K. P. (2012). The ideology of pragmatism: neo-liberal globalisation and political authoritarianism in Singapore. *J. Contemp. Asia* 42, 67–92. doi: 10.1080/00472336.2012.634644

Tansel, C. (2017). "Authoritarian neoliberalism," in *States of Discipline: Authoritarian Neoliberalism and the Contested Reproduction of Capitalist Order,* ed C. Tansel (Lanham, MD: Rowman and Littlefield), 1–28.

Thompson, M. R. (2019). "Singapore and the lineages of authoritarian modernity in East Asia," in *The Limits of Authoritarian Governance in Singapore's Developmental State*, eds L. Z. Rahim and M. Barr (Singapore: Springer Singapore), 29–48. doi: 10.1057/978-1-137-51167-6

Vosko, L. F. (Ed.). (2006). *Precarious Employment: Understanding Labour Market Insecurity in Canada*. Montreal, QC: McGill-Queen's University Press. 43–66.

Yea, S. (2017) The art of not being caught: temporal strategies for disciplining unfree labour in Singapore's contract migration. *Geoforum* 78, 179–188. doi: 10.1016/j.geoforum.2016.05.003

Yea, S., and Chok, S. (2018). Unfreedom unbound: developing a cumulative approach to understanding unfree labour in Singapore. *Work Employ. Soc.* 32, 925–941. doi: 10.1177/0950017017738956

Conflict of Interest: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling editor declared co-authorships with the author.

frontiers in Public Health

BRIEF RESEARCH REPORT
published: 21 August 2020
doi: 10.3389/fpubh.2020.00497

Overview of the First 6 Months of Clinical Trials for COVID-19 Pharmacotherapy: The Most Studied Drugs

Maria Laura Idda[1], *Dorian Soru*[2] *and Matteo Floris*[1,3]*

[1] Institute for Genetic and Biomedical Research, National Research Council, Sassari, Italy, [2] Independent Consultant, Ovodda, Italy, [3] Department of Biomedical Sciences, University of Sassari, Sassari, Italy

SARS-CoV-2 rapidly spread from China until it was defined a pandemic by WHO in March 2020. Related scientific papers have rapidly extended information regarding the diagnosis, treatment and epidemiology of COVID-19 infection. To date, no vaccine or definitive treatment is available to defeat the virus and therapies are mainly based on existing drugs used to treat other conditions. Existing therapies used in several clinical trials work by affecting the biology of COVID-19 and/or counteracting the harmful host excessive immune response. Here, we have reviewed 526 ongoing clinical trials for COVID-19 to provide a perspective on the first 6 months of global efforts to identify an effective therapy. The drugs most actively tested in various centers include hydroxychloroquine, ritonavir, azithromycin, tocilizumab, lopinavir chloroquine and ivermectin. Our analysis shows that most clinical trials focus on a small number of candidate drugs (namely hydroxychloroquine and chloroquine representing 25% of total clinical trials) while underestimating the potential of other promising drugs. A global coordination in clinical trial management could avoid duplications and increase the effectiveness of the response to the global challenge.

Keywords: clinical trial (2.172), COVID-19, coronavirus (2019-nCoV), COVID-19 (condition), COVID-19 infection

OPEN ACCESS

Edited by:
Alexander Rodriguez-Palacios, Case Western Reserve University, United States

Reviewed by:
Nitya Singh, University of Florida, United States
Hong-Qiu Gu, National Clinical Research Center for Neurological Diseases, China

***Correspondence:**
Matteo Floris
mfloris@uniss.it

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Public Health

Received: *22 April 2020*
Accepted: *31 July 2020*
Published: *21 August 2020*

Citation:
Idda ML, Soru D and Floris M (2020) Overview of the First 6 Months of Clinical Trials for COVID-19 Pharmacotherapy: The Most Studied Drugs. Front. Public Health 8:497. doi: 10.3389/fpubh.2020.00497

INTRODUCTION

The outbreak of the coronavirus disease 2019 (COVID-19) has generated a global health issue. COVID-19 is a pathogenic viral infection caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which appeared in 2019 in Wuhan, China. From a pathological point of view, the most common symptoms observed during COVID-19 infection are fever (83.3%), cough (60.3%), dyspnea, and myalgia or fatigue; and anosmia and ageusia are also commonly observed (1, 2). Furthermore, gastrointestinal symptoms could also be initial manifestations of COVID-19 and contribute to the diffusion of the virus through fecal samples, especially in children (3). More recently, development of venous thromboembolism in patients with COVID-19 has been reported (4).

SARS-CoV-2 is a betacoronavirus, one of the four genera of coronaviruses, belonging to the same sub-group as the Severe Acute Respiratory Syndrome-CoV (SARS-CoV, SARS outbreak in 2002) and the Middle East Respiratory Syndrome-CoV (MERS-CoV, MERS outbreak in 2012) (5). Generally, coronaviruses are extremely small (65–125 nm in diameter) and contain a

single-stranded RNA ∼26–32 Kbs long (6). All coronavirus genomes are organized as follows: a 5′-untranslated region (5′-UTR), open reading frame (orf) 1a/b encoding proteins necessary for virus replication, downstream genes encoding structural proteins including *spike*, and elements necessary for the envelop, membrane, and nucleocapsid production; finally, accessory proteins and the 3′-untranslated region (3′-UTR) (7). *Spike* is a glycoprotein located on the outer surface of coronaviruses that is responsible for the attachment and entry of the virus to host cells. After binding of *spike* to the human receptor angiotensin-converting enzyme 2 gene (ACE2), a conformational change in the spike protein facilitates the fusion of the viral envelope with the cell membrane through the endosomal pathway (8). Then SARS-CoV-2 releases RNA into the host cell. Genome encoding begin following RNA entering to the host cell and enables the expression of proteins, which progress the adaptation of the virus to the human host. Importantly, the entry mechanism of coronavirus is strongly dependent on cellular proteases. For coronavirus such as the SARS-CoV, the transmembrane protease serine 2 (TMPRSS2) and cathepsin play a critical role in virus entry, they split the *spike* protein and begin all the changes necessary for the virus penetration (9). Recently it was reported that SARS-CoV-2 may use a similar mechanism and that SARS-CoV-2 cell entry may be facilitated by ACE2 and TMPRSS2 (10, 11).

In addition to a growing knowledge of molecular mechanisms, new information regarding diagnosis, treatment and epidemiology of COVID is rapidly accumulating, permitting greater understanding of the disease pathway and progression and identification of new pharmacological targets. While numerous clinical trials are on-going to identify therapeutic approaches by repurposing existing drugs, today the main international response to COVID-19 is mainly limited to contain disease spread. The need to identify innovative treatment strategies remains a priority.

Here, we reviewed 526 ongoing clinical trials (last update: July 6, 2020) to offer a view on the first 6 months of efforts to identify an effective therapy for COVID-19. A large number of drugs (265) are under investigation, but current efforts are biased toward a limited number of them. Indeed, the great majority of clinical trials are focused on a small number of candidate drugs including, hydroxychloroquine, ritonavir, azithromycin, tocilizumab, lopinavir chloroquine and ivermectin (12), while potentially and more promising ones are less considered. For example, host-directed therapies such as those based on inhibitors of the human serine protease TMPRSS2 (bromhexine, camostat, and nafamostat) are considerably less explored. Conversely, there are conflicting and discordant results on hydroxychloroquine, the most tested drug (about 1 of 5 trials). Global coordination of clinical trials could avoid current redundancy and potentiate the effort to explore other possibilities.

RESULTS AND DISCUSSION

The current COVID-19 pandemic boosted the growth of new pharmaceutical research programs and the proliferation of a large number of clinical trials worldwide. Indeed, researchers are attempting to identify drugs to treat the disease using different approaches including repurposing of existing drugs, high throughput screening and virtual screening of new compound; the use of natural and traditional products have also been evaluated. Repurposing of existing drugs, the identification of a new medical use, in this case antiviral activity, for already known drugs, including approved, and discontinued one, is playing a key role in this effort. Initially, interferons nebulization and anti-viral drugs were used to reduce the viral load. Type I interferons (IFNs) inhibit the replication of both DNA and RNA viruses at different stages of their replicative cycles and have strong antiviral activity (13, 14). Unfortunately, only remdesivir, an antiviral drug with nucleotide analog activity has demonstrated relevant antiviral activity. Preliminary observations from a multicentric study, in a cohort of 53 patients hospitalized for severe Covid-19 who were treated with compassionate-use remdesivir, demonstrated clinical improvement in 68% of patients (15). More recently, a larger double-blind, randomized, placebo-controlled trial demonstrated that intravenous remdesivir is superior to placebo in shortening the time to recovery in adults hospitalized with Covid-19. Furthermore, the same study estimated that 14-days mortality was 7.1% with remdesivir and 11.9% with placebo (16).

To have a complete picture of the ongoing trials to treat COVID-19 infection, we collected a comprehensive list of COVID-19 clinical trials from the 2 main public repositories, as of July 6th, 2020 (**Methods**). We then made coherent the names of the drugs (for example, different salts of the same active principle were considered as one single drug) provided by the different sources (**Methods**) and obtained a final list of 526 clinical trials that were analyzed. Most trials focus on a restricted number of drugs, including hydroxychloroquine and antivirals previously used for treatment of other viral infection, mainly HIV (**Figure 1** and **Table 1**). Of note is the use of anti - inflammatory molecules which prevent adverse effects related to over-reactive immune system.

Hydroxycloroquine ($N = 106$ clinical trials), Azithromycin ($N = 33$), the antiviral compounds Ritonavir and Lopinavir ($N = 33$ and 29 clinical trials, respectively), and Tocilizumab ($N = 29$) are among the drugs more actively tested. Ritonavir and Lopinavir – a classical HIV first-line therapy - are usually administered in combination. They are followed by Chloroquine ($N = 25$ trials) and Ivermectin ($N = 24$). The distribution of the number of clinical trials per drug is significantly skewed toward such low number of drugs ($p < 0.001$, 1 DF Chi square test).

Chloroquine and its derivative Hydroxychloroquine are widely used to treat malarial infection and selected inflammatory conditions such as autoimmune disease (25). Multiple lines of evidence have suggested that chloroquine has the capacity to inhibit the replication of several micro-organisms, including coronaviruses such as SARS-CoV-2, *in vitro* (17). Today, hydroxychloroquine and chloroquine are under investigation in clinical trials for both, prophylaxis in pre-exposure to virus and treatment post-exposure to SARS-CoV-2 (26). Many hospitals are currently using hydroxychloroquine as first-line therapy for hospitalized patients with COVID-19,

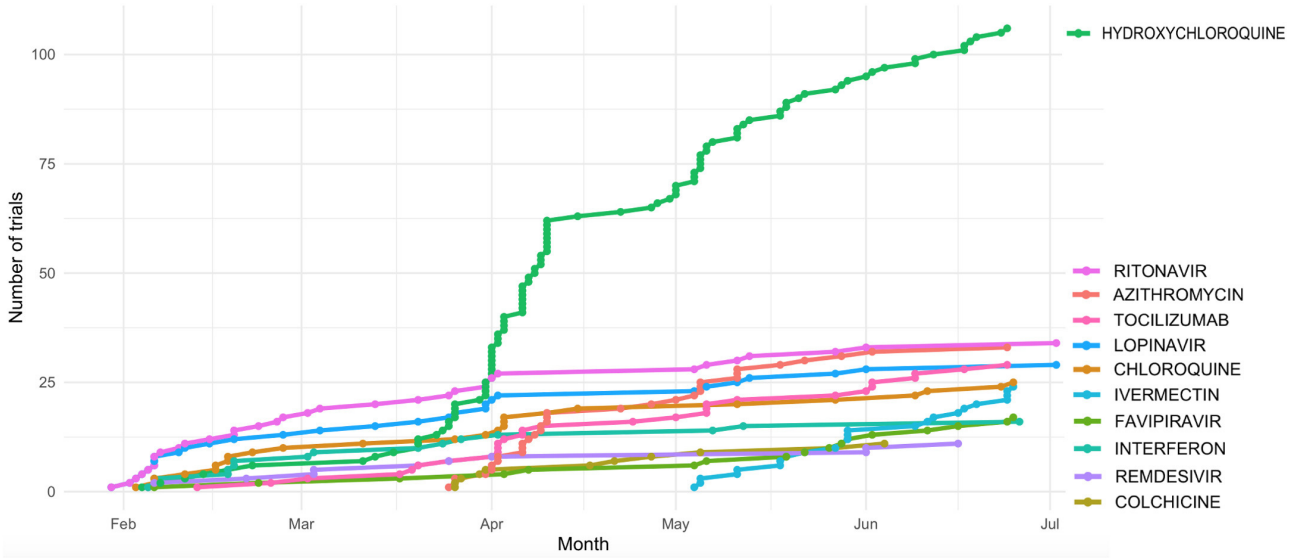


FIGURE 1 | Cumulative number of clinical trials of the most actively tested drugs registered during the first 6 Months after the first COVID-19 published trial (23 Jan 2020).

and on March 29 FDA issued authorization for 30 million doses of hydroxychloroquine and chloroquine donated by Sandoz. Unfortunately, clinical data supporting the effectiveness of these two drugs are still inconclusive. The efficacy of hydroxychloroquine was supported by a small trial with 62 patients suffering from severe COVID-19 diagnosed and admitted to Renmin Hospital of Wuhan University (27). Later, a smaller pilot study at the Shanghai Public Health Clinical Center (28) demonstrated its activity against SARS-CoV-2, although its use was subsequently discouraged by a smaller study with just 11 patients from a clinical study performed in a French hospital (29). Beyond the lack of data on the real effectiveness of these drugs until the middle of July, the possibility of side effects as a result of their use is well-known, especially when provided in combination with other drugs. A group of cardiologists in New York, for example, found notable signs of QT interval prolongation in 30% in a group of 84 COVID-19 patients treated with hydroxychloroquine and azithromycin (30).

The main antiretroviral drugs studied in the world against COVID-19 are Ritonavir and Lopinavir, two antivirals often used in combination as first-line therapy against HIV. Interestingly, the largest study in hospitalized adult patients with severe Covid-19 has shown no benefit as compared with standard care after lopinavir –ritonavir treatment (20). Although, even in this case the data supporting the efficacy are unfavorable, regulatory agencies have approved the use of this combination therapy, limiting it to less severe COVID-19 patients [1].

[1]Available online at: http://www.salute.gov.it/portale/news/p3_2_1_1_1.jsp?lingua=italiano&menu=notizie&p=dalministero&id=4395.

Other antiviral compounds among the most tested drugs are Favipiravir, Umifenovir and Oseltamivir. Favipiravir has been approved in Japan and China for the treatment of novel influenza virus infections; its efficacy has been only weakly documented by a paper later retracted (31) and by a preprint article (32). Umifenovir (trade name: Arbidol) is a dual-acting direct antiviral/host-targeting agent (33); it is under evaluation in 12 clinical trials, and to date only 2 small-scale studies tested its efficacy in comparison with a Lopinavir/Ritonavir based treatment (34, 35). Finally, during a clinical trial to test the effectiveness of Oseltamivir the authors noted no favorable outcomes against SARS-CoV-2 (36).

As mentioned above, the most promising antiviral compound tested for COVID-19 is Remdesivir.

The immunosuppressant anti-IL6 Tocilizumab (37), used for the treatment of rheumatoid arthritis, is the most widely tested drug directed against a human target. Several reports have identified elevation of IL-6 levels in critically ill COVID-19 patients, as compared with that of survivors and those with less severe disease (1). Consistent with this finding and with the efficacy to restrict the IL-6 pathway, Tocilizumab is tested in 29 trials. Tocilizumab is approved for the treatment of severe or life-threatening cytokine-release syndrome caused by chimeric antigen receptor T-cell therapy (37). Additionally, tocilizumab also has FDA-approved indications for giant cell arteritis, polyarticular juvenile idiopathic arthritis, and systematic juvenile idiopathic arthritis. Until now, tocilizumab was not officially approved by the FDA for use in COVID-19 treatment and few published data pertain to the safety or efficacy of this drug for COVID therapy. Other immunosuppressive agents, Anakinra (anti-IL1) and Sarilumab (anti-IL6 receptor) are being tested in 8 and 5 different trials, respectively.

TABLE 1 | Description of the drugs tested in at least 10 COVID-19 clinical trials as of July 8, 2020.

Drug	Number of trials	Description	References
HYDROXYCHLOROQUINE	106	Treatment of uncomplicated malaria, rheumatoid arthritis, chronic discoid lupus erythematosus, and systemic lupus erythematosus. Hydroxychloroquine accumulation in human organelles also raise their pH, which inhibits antigen processing, prevents the alpha and beta chains of the major histocompatibility complex (MHC) class II from dimerizing, inhibits antigen presentation of the cell, and reduces the inflammatory response. The raised pH in endosomes, prevent virus particles (such as SARS-CoV and SARS-CoV-2) from utilizing their activity for fusion and entry into the cell	(17)
RITONAVIR	33	HIV protease inhibitor that interferes with the reproductive cycle of HIV; more commonly used as a booster of other protease inhibitors. For example, Ritonavir is a potent inhibitor of the enzymes responsible for lopinavir metabolism, and its co-administration "boosts" lopinavir exposure and improves antiviral activity	(18)
AZITHROMYCIN	33	Antibiotic used for the treatment of a number of bacterial infections	
TOCILIZUMAB	29	Recombinant, humanized, anti-human interleukin 6 (IL-6) receptor monoclonal antibody	(19)
LOPINAVIR	29	Antiretroviral protease inhibitor used in combination with other antiretrovirals in the treatment of HIV-1 infection	(20)
CHLOROQUINE	25	See HYDROXYCHLOROQUINE	
IVERMECTIN	24	This drug has a broad-spectrum activity with high lipid solubility and possesses numerous effects on parasites, nematodes, arthropods, flavivirus, mycobacteria, and mammals through a variety of mechanisms	(21)
FAVIPIRAVIR	17	A pyrazine analog initially approved for therapeutic use in resistant cases of influenza. The antiviral targets RNA-dependent RNA polymerase (RdRp) enzymes, which are necessary for the transcription and replication of viral genomes	(22)
INTERFERON	16	First cytokines produced during a viral infection; inflammation, signaling and immunomodulation	(23)
REMDESIVIR	11	Remdesivir is a nucleoside analog that is expected to inhibit the action of RNA polymerase	
COLCHICINE	11	Inhibits the hepatitis C NS5B protein, RNA-dependent RNA polymerase	(24)

Surprisingly, none of the drugs directed against the mechanism of viral entry into human cells are among the most tested drugs. In particular, we observed only 5 and 4 trials for the serine protease inhibitors Camostat and Bromhexine, respectively, and 1 single trial for the analog Nafamostat (**Supplemental Table S1**).

We also identified several clinical trials where drug-drug interaction alerts should be considered when the combinations are proposed in the same trial (**Table 2**). Among others, Hydroxychloroquine (HCQ) is frequently tested with Lopinavir (which increases the serum levels of HCQ) and with Ritonavir (whose serum levels are increased by the concomitant administration of HCQ).

Based on the current data, it is evident that mainly repurposed antiviral drugs (whose function is not yet guaranteed) are tested for COVID-19 treatment. Interestingly, drugs directed against the virus entry and replication mechanism (including in particular host-directed-therapies such as *nafamostat mesylate* and the analogous *camostat mesylate*, or the *recombinant ACE2 protein*) are tested less frequently, although they have a mechanism of action intimately and directly involved in the biology of the infection.

The use-abuse of repurposing could be one of the main reason for COVI-19 trials failure. What is sure, especially in clinical trials, is that good results must be obtained with slow and careful experiments to be reliable and secure for population. Remdesivir for example, initially developed against hepatitis C, which showed great potential against zoonotic viruses including SARS and MERS, have been found to help COVID-19 patients to recover faster. The drug did not work against hepatitis C as expected but researchers established that Remdesivir is safe for humans. Thus, after COVID-19 outbreak, researchers could quickly roll out clinical trials to test Remdesivir for Covid-19. This example clearly shows that the reason why most clinical trials are looking to repurpose existing drugs is mainly related to the possibility to faster use them for human patients escaping months or years of safety testing.

Another reason of these failures could be the existence of a perverse mechanism, where the choice of priorities in drug testing is led by small uncontrolled studies that fuelled a strong

TABLE 2 | Drug combinations tested in clinical trials where drug-drug interaction alerts are reported in the Drugbank "Drug-*Drug Interaction Checker.*"

Drug A	Effects	Drug B
Azithromycin	Increases risk or severity of QTc prolongation of	Hydroxychloroquine
Daclatasvir	Increases serum levels of	Sofosbuvir
Favipiravir	Lowers metabolism rate of	Chloroquine
Hydroxychloroquine	Increases serum levels of	Ritonavir
Lopinavir	Lowers excretion rate & increases serum levels of	Emtricitabine
Lopinavir	Increases serum levels of	Hydroxychloroquine
Ritonavir	Lowers excretion rate & increases serum levels of	Sofosbuvir

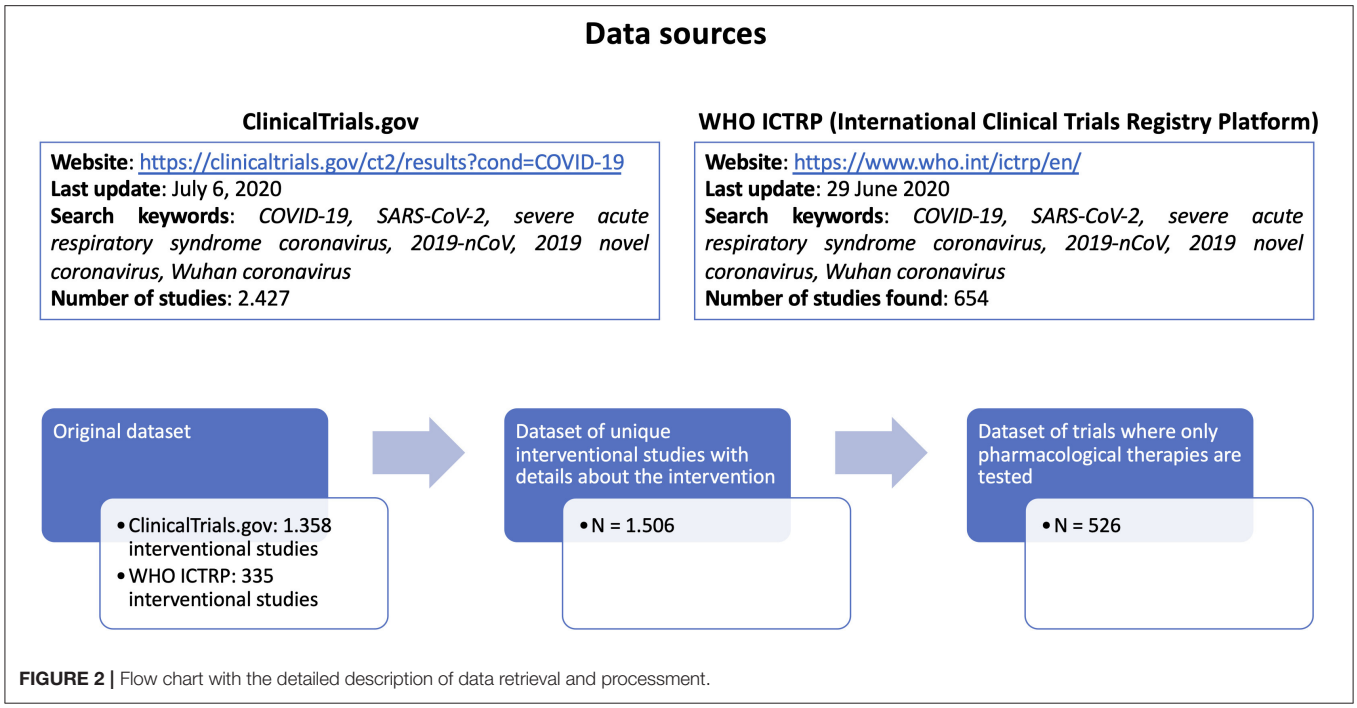


FIGURE 2 | Flow chart with the detailed description of data retrieval and processment.

pressure by media, politicians and not by strong scientific evidences strongly contribute to this problem. Indeed, 6 months after the first clinical trial the number of coronavirus cases are still rising and nothing seems to be able to work as an effective Covid-19 treatment.

This obvious bias, generating overlapping studies, could only be overcome by global coordination of clinical trial policies, which could also help to avoid redundancy that also slows the identification of effective therapies.

METHODS

Data Collection

We collected a comprehensive list of COVID-19 clinical trials from 2 different public repositories (ClinicalTrials.gov and WHO ICTRP - International Clinical Trials Registry Platform, both accessed on July the 6th, 2020), using the search keywords "COVID-19, SARS-CoV-2, severe acute respiratory syndrome coronavirus, 2019-nCoV, 2019 novel coronavirus, Wuhan coronavirus," and considering only pharmacological interventions where one or more drugs are explicitly listed. From ClinicalTrials.gov (https://clinicaltrials.gov/ct2/results?cond=COVID-19) we retrieved 2.427 studies, of which 1.358 were interventional studies. From WHO ICTRP (International Clinical Trials Registry Platform, https://www.who.int/ictrp/en/) we retrieved 654 studies, of which 335 were interventional studies.

Based on the identifiers assigned to each trial, duplicate entries were considered only once.

The merged dataset contained 1.693 unique interventional studies, of which, 1.506 have complete intervention details (**Figure 2**).

Analysis

From the raw data we kept only trials where the following informations were clearly available: trial recruitment status, list of participating countries, name of the drug(s), clinical trial phase. We then performed a standardization of the information provided by the different sources. Furthermore, for each active ingredient we retrieved the corresponding DrugBank identifier (38) to retrieve drug-related informations.

We excluded from our analysis all the therapies whose active ingredients were not clearly declared, and therapies based on nutraceuticals and traditional medications.

We obtained a final list of 526 clinical trials considered for the analysis (**Supplemental Table S1**).

The non-random nature of the distribution of number of trials per drug has been checked with a 1DF chi square test using the R package for statistical analysis, that returned a $p < 0.001$.

Considering that many drugs are tested in combinations, we checked whether the concomitant administration of these drug could be problematic using the *Drug-Drug Interaction Checker* [https://www.drugbank.ca/interax/multi_search], a freely available resource reporting data from clinical guidelines, labels and scientific literature, and covering approved drugs by the Food and Drug Administration (FDA), Health Canada and the European Medical Association (EMA).

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

MI: conceptualization and writing–original draft. DS: data curation. MF: conceptualization, writing–original draft, and supervision. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2020.00497/full#supplementary-material

REFERENCES

1. Fu L, Wang B, Yuan T, Chen X, Ao Y, Fitzpatrick T, et al. Clinical characteristics of coronavirus disease 2019 (COVID-19) in China: a systematic review and meta-analysis. *J Infect.* (2020) 80:656–65. doi: 10.1016/j.jinf.2020.03.041
2. Vaira LA, Salzano G, Deiana G, De Riu G. Anosmia and ageusia: common findings in COVID-19 patients. *Laryngoscope.* (2020) 130:1787. doi: 10.1002/lary.28692
3. Gu J, Han B, Wang J. COVID-19: gastrointestinal manifestations and potential fecal-oral transmission. *Gastroenterology.* (2020) 158:1518–9. doi: 10.1053/j.gastro.2020.02.054
4. Wang T, Chen R, Liu C, Liang W, Guan W, Tang R, et al. Attention should be paid to venous thromboembolism prophylaxis in the management of COVID-19. *Lancet Haematol.* (2020) 7:e362–3. doi: 10.1016/S2352-3026(20)30109-5
5. Lai CC, Shih TP, Ko WC, Tang HJ, Hsueh PR. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and coronavirus disease-2019 (COVID-19): the epidemic and the challenges. *Int J Antimicrob Agents.* (2020) 55:105924. doi: 10.1016/j.ijantimicag.2020.105924
6. Shereen MA, Khan S, Kazmi A, Bashir N, Siddique R. COVID-19 infection: origin, transmission, and characteristics of human coronaviruses. *J Adv Res.* (2020) 24:91–8. doi: 10.1016/j.jare.2020.03.005
7. Luk HKH, Li X, Fung J, Lau SKP, Woo PCY. Molecular epidemiology, evolution and phylogeny of SARS coronavirus. *Infect Genet Evol.* (2019) 71:21–30. doi: 10.1016/j.meegid.2019.03.001
8. Hussain M, Jabeen N, Raza F, Shabbir S, Baig AA, Amanullah A, et al. Structural variations in human ACE2 may influence its binding with SARS-CoV-2 spike protein. *J Med Virol.* (2020). doi: 10.1002/jmv.25832. [Epub ahead of print].
9. Wu F, Zhao S, Yu B, Chen YM, Wang W, Song ZG, et al. A new coronavirus associated with human respiratory disease in China. *Nature.* (2020) 579:265–9. doi: 10.1038/s41586-020-2008-3
10. Hoffmann M, Kleine-Weber H, Schroeder S, Krüger N, Herrler T, Erichsen S, et al. SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor. *Cell.* (2020) 181:271–80.e8. doi: 10.1016/j.cell.2020.02.052
11. Zhou P, Yang XL, Wang XG, Hu B, Zhang L, Zhang W, et al. A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature.* (2020) 579:270–3. doi: 10.1038/s41586-020-2012-7
12. Sanders JM, Monogue ML, Jodlowski TZ, Cutrell JB. Pharmacologic treatments for coronavirus disease 2019 (COVID-19): a review. *JAMA.* (2020) 323:1824–36. doi: 10.1001/jama.2020.6019
13. Wang BX, Fish EN. Global virus outbreaks: Interferons as 1st responders. *Semin Immunol.* (2019) 43:101300. doi: 10.1016/j.smim.2019.101300
14. Barlow A, Landolf KM, Barlow B, Yeung SYA, Heavner JJ, Claassen CW, et al. Review of emerging pharmacotherapy for the treatment of coronavirus disease 2019. *Pharmacotherapy.* (2020) 40:416–37. doi: 10.1002/phar.2398
15. Grein J, Ohmagari N, Shin D, Diaz G, Asperges E, Castagna A, et al. Compassionate use of remdesivir for patients with severe Covid-19. *N Engl J Med.* (2020) 382:2327–36. doi: 10.1056/NEJMoa2007016
16. Beigel JH, Tomashek KM, Dodd LE, Mehta AK, Zingman BS, Kalil AC, et al. Remdesivir for the treatment of Covid-19 - preliminary report. *N Engl J Med.* (2020). doi: 10.1056/NEJMc2022236. [Epub ahead of print].
17. Liu J, Cao R, Xu M, Wang X, Zhang H, Hu H, et al. Hydroxychloroquine, a less toxic derivative of chloroquine, is effective in inhibiting SARS-CoV-2 infection in vitro. *Cell Discov.* (2020) 6:16. doi: 10.1038/s41421-020-0156-0
18. Choy KT, Wong AY, Kaewpreedee P, Sia SF, Chen D, Yan Hui KP, et al. Remdesivir, lopinavir, emetine, and homoharringtonine inhibit SARS-CoV-2 replication *in vitro*. *Antiviral Res.* (2020) 178:104786. doi: 10.1016/j.antiviral.2020.104786
19. Mehta P, McAuley DF, Brown M, Sanchez E, Tattersall RS, Manson JJ, et al. COVID-19: consider cytokine storm syndromes and immunosuppression. *Lancet.* (2020) 395:1033–4. doi: 10.1016/S0140-6736(20)30628-0
20. Cao B, Wang Y, Wen D, Liu W, Wang J, Fan G, et al. A trial of lopinavir-ritonavir in adults hospitalized with severe Covid-19. *N Engl J Med.* (2020) 382:1787–99. doi: 10.1056/NEJMoa2001282
21. Heidary F, Gharebaghi R. Ivermectin: a systematic review from antiviral effects to COVID-19 complementary regimen. *J Antibiot.* (2020) 73:593–602. doi: 10.1038/s41429-020-0336-z
22. Dong L, Hu S, Gao J. Discovering drugs to treat coronavirus disease 2019 (COVID-19). *Drug Discov Ther.* (2020) 14:58–60. doi: 10.5582/ddt.2020.01012
23. Sallard E, Lescure FX, Yazdanpanah Y, Mentre F, Peiffer-Smadja N. Type 1 interferons as a potential treatment against COVID-19. *Antiviral Res.* (2020) 178:104791. doi: 10.1016/j.antiviral.2020.104791
24. Elfiky AA. Anti-HCV, nucleotide inhibitors, repurposing against COVID-19. *Life Sci.* (2020) 248:117477. doi: 10.1016/j.lfs.2020.117477
25. Haładyj E, Sikora M, Felis-Giemza A, Olesinska M. Antimalarials - are they effective and safe in rheumatic diseases? *Reumatologia.* (2018) 56:164–73. doi: 10.5114/reum.2018.76904
26. *CDC - Information for Clinicians on Investigational Therapeutics for Patients with COVID-19.* Available online at: https://www.cdc.gov/coronavirus/2019-ncov/hcp/therapeutic-options.html (accessed July 06, 2020).
27. Chen Z, Hu J, Zhang Z, Jiang S, Han S, Yan D, et al. Efficacy of hydroxychloroquine in patients with COVID-19: results of a randomized clinical trial. *medRxiv.* (2020). doi: 10.1101/2020.03.22.20040758
28. Chen J, Liu D, Liu L, Liu P, Xu Q, Xia L, et al. A pilot study of hydroxychloroquine in treatment of patients with common coronavirus disease-19 (COVID-19). *J Zhejiang Univ.* (2020) 49:215–9. doi: 10.3785/j.issn.1008-9292.2020.03.03
29. Molina JM, Delaugerre C, Goff JL, Mela-Lima B, Ponscarme D, Goldwirt L, et al. No Evidence of rapid antiviral clearance or clinical benefit with the combination of hydroxychloroquine and azithromycin in patients with severe COVID-19 infection. *Méd Malad Infect.* (2020) 50:384. doi: 10.1016/j.medmal.2020.03.006
30. Chorin E, Dai M, Shulman E, Wadhwani L, Cohen RB, Barbhaiya C, et al. The QT interval in patients with SARS-CoV-2 infection treated with hydroxychloroquine/azithromycin. *medRxiv.* (2020). doi: 10.1101/2020.04.02.20047050
31. Cai Q, Yang M, Liu D, Chen J, Shu D, Xia J, et al. Experimental treatment with Favipiravir for COVID-19: an open-label control study. *Engineering.* (2020). doi: 10.1016/j.eng.2020.03.007. [Epub ahead of print].
32. Chen C, Huang J, Yin P, Zhang Y, Cheng Z, Wu J, et al. Favipiravir versus arbidol for COVID-19: a randomized clinical trial. *medRxiv.* (2020). doi: 10.1101/2020.03.17.20037432
33. Blaising J, Polyak SJ, Pécheur EI. Arbidol as a broad-spectrum antiviral: an update. *Antiviral Res.* (2014) 107:84–94. doi: 10.1016/j.antiviral.2014

34. Zhu Z, Lu Z, Xu T, Chen C, Yang G, Zha T, et al. Arbidol monotherapy is superior to lopinavir/ritonavir in treating COVID-19. *J Infect.* (2020) 81:e21–3. doi: 10.1016/j.jinf.2020.03.060
35. Deng L, Li C, Zeng Q, Liu X, Li X, Zhang H, et al. Arbidol combined with LPV/r versus LPV/r alone against corona virus disease 2019: a retrospective cohort study. *J Infect*. (2020) 81:e1–5. doi: 10.1016/j.jinf.2020.03.002
36. Wang D, Hu B, Hu C, Zhu F, Liu X, Zhang J, et al. Clinical characteristics of 138 hospitalized patients with 2019. Novel coronavirus-infected pneumonia in Wuhan, China. *JAMA*. (2020) 323:1061–9. doi: 10.1001/jama.2020.1585
37. Zhang C, Wu Z, Li JW, Zhao H, Wang GQ. The cytokine release syndrome (CRS) of severe COVID-19 and Interleukin-6 receptor (IL-6R) antagonist Tocilizumab may be the key to reduce the mortality. *Int J Antimicrob Agents*. (2020) 29:105954. doi: 10.1016/j.ijantimicag.2020.105954
38. Wishart DS, Feunang YD, Guo AC, Lo EJ, Marcu A, Grant JR, et al. DrugBank 5.0: a major update to the DrugBank database for 2018. *Nucleic Acids Res.* (2017) 46:D1074–82. doi: 10.1093/nar/gkx1037

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Medicine

ORIGINAL RESEARCH
published: 21 August 2020
doi: 10.3389/fmed.2020.00541

Lung Mechanics of Mechanically Ventilated Patients With COVID-19: Analytics With High-Granularity Ventilator Waveform Data

Huiqing Ge[1†], Qing Pan[2†], Yong Zhou[3], Peifeng Xu[1], Lingwei Zhang[2], Junli Zhang[4], Jun Yi[5], Changming Yang[6], Yuhan Zhou[2], Limin Liu[7] and Zhongheng Zhang[8]**

[1] Department of Respiratory Care, Sir Run Run Shaw Hospital, Zhejiang University School of Medicine, Hangzhou, China, [2] College of Information Engineering, Zhejiang University of Technology, Hangzhou, China, [3] Department of Pulmonary Disease, Sir Run Run Shaw Hospital, Zhejiang University School of Medicine, Hangzhou, China, [4] Department of Infectious Diseases, Sir Run Run Shaw Hospital, Zhejiang University School of Medicine, Hangzhou, China, [5] Thoracic Cardiovascular Surgery, Jingmen First People's Hospital, Jingmen, China, [6] Department of Anesthesiology, The First People's of Hospital of Jingmen City, Jingmen, China, [7] Department of Administration, Sir Run Run Shaw Hospital, Zhejiang University School of Medicine, Hangzhou, China, [8] Department of Emergency Medicine, Sir Run Run Shaw Hospital, Zhejiang University School of Medicine, Hangzhou, China*

OPEN ACCESS

Edited by:
Laurent Pierre Nicod, University of Lausanne, Switzerland

Reviewed by:
Pai-Chien Chou, Taipei Medical University Hospital, Taiwan
Pan Pan, People's Liberation Army General Hospital, China

***Correspondence:**
Limin Liu
llmin@zju.edu.cn
Zhongheng Zhang
zh_zhang1984@zju.edu.cn

† These authors have contributed equally to this work

Specialty section:
This article was submitted to Pulmonary Medicine, a section of the journal Frontiers in Medicine

Received: *07 May 2020*
Accepted: *30 July 2020*
Published: *21 August 2020*

Citation:
Ge H, Pan Q, Zhou Y, Xu P, Zhang L, Zhang J, Yi J, Yang C, Zhou Y, Liu L and Zhang Z (2020) Lung Mechanics of Mechanically Ventilated Patients With COVID-19: Analytics With High-Granularity Ventilator Waveform Data. Front. Med. 7:541. doi: 10.3389/fmed.2020.00541

Background: Lung mechanics during invasive mechanical ventilation (IMV) for both prognostic and therapeutic implications; however, the full trajectory lung mechanics has never been described for novel coronavirus disease 2019 (COVID-19) patients requiring IMV. The study aimed to describe the full trajectory of lung mechanics of mechanically ventilated COVID-19 patients. The clinical and ventilator setting that can influence patient-ventilator asynchrony (PVA) and compliance were explored. Post-extubation spirometry test was performed to assess the pulmonary function after COVID-19 induced ARDS.

Methods: This was a retrospective study conducted in a tertiary care hospital. All patients with IMV due to COVID-19 induced ARDS were included. High-granularity ventilator waveforms were analyzed with deep learning algorithm to obtain PVAs. Asynchrony index (AI) was calculated as the number of asynchronous events divided by the number of ventilator cycles and wasted efforts. Mortality was recorded as the vital status on hospital discharge.

Results: A total of 3,923,450 respiratory cycles in 2,778 h were analyzed (average: 24 cycles/min) for seven patients. Higher plateau pressure (Coefficient: −0.90; 95% CI: −1.02 to −0.78) and neuromuscular blockades (Coefficient: −6.54; 95% CI: −9.92 to −3.16) were associated with lower AI. Survivors showed increasing compliance over time, whereas non-survivors showed persistently low compliance. Recruitment maneuver was not able to improve lung compliance. Patients were on supine position in 1,422 h (51%), followed by prone positioning (499 h, 18%), left positioning (453 h, 16%), and right positioning (404 h, 15%). As compared with supine positioning, prone positioning was associated with 2.31 ml/cmH_2O (95% CI: 1.75 to 2.86; $p < 0.001$) increase in lung compliance. Spirometry tests showed that pulmonary functions were reduced to one third of the predicted values after extubation.

Conclusions: The study for the first time described full trajectory of lung mechanics of patients with COVID-19. The result showed that prone positioning was associated with improved compliance; higher plateau pressure and use of neuromuscular blockades were associated with lower risk of AI.

Keywords: COVID-19, lung mechanics, mechanical ventilation, asynchrony, asynchonized, prone positioning

INTRODUCTION

The novel coronavirus disease 2019 (COVID-19) imposes an important and urgent threat to global health (1, 2). A substantial proportion of COVID-19 cases will develop severe acute respiratory distress syndrome (ARDS) that requires invasive mechanical ventilation (IMV). The mortality rate of such patients can be as high as 40% (3), depending on comorbidities and the available medical resources. Mechanical ventilation is an important strategy to treat such patients; and lung mechanics can have both prognostic and therapeutic implications. Lung compliance is an important mechanical parameter that should be monitored during IMV. For example, lung recruitment maneuver (RM) has been used to improve lung compliance in severe ARDS (4). There is also evidence in general ARDS population that poor lung compliance without improvement during IMV is associated with poor clinical outcome (5). Patient ventilator asynchrony (PVA) is another important parameter that should be stressed during IMV. Risk factors of PVA has been widely investigated, including hours of the day, use of sedatives, ventilation mode and tidal volume (6, 7). While several studies showed that PVA was associated with clinical outcome, others did not (8, 9). There is preliminary opinion suggesting that lung mechanics of COVID-19 induced ARDS can be quite different from general ARDS (10). However, there is no empirical data on the lung mechanics in COVID-19 patients on IMV. Furthermore, previous studies are limited in several aspects. First, there is no continuous pulmonary mechanics evaluation, including the response of lung recruitment during IMV, all events during prone ventilation. Second, most techniques for the detection of PVA and other parameters requires physical presence of an expert physician at the bedside and is thus only feasible during short periods (11–13). In addition, most studies explored risk factors for PVA in a fixed-time model (14). In reality, both risk factors and PVA and compliance were time-varying (15).

In order to make this gap end, the purpose of the study were 4-folds: (1) to describe the lung mechanics of COVID-19 patients by analyzing high-granularity ventilator waveform data; (2) to explore whether the lung compliance can be influenced by clinical factors, such as recruitment maneuver (RM) and body positioning; (3) to identify risk factors for PVA during IMV in COVID-19 patients; and (4) To describe post-extubation lung functions for survivors with spirometry test.

Abbreviations: AI, asynchrony index; WOB, work of breathing; PEEP, positive end expiratory pressure; DT, delayed triggering; IEE, ineffective effort during expiration; IQR, interquartile range; COVID-19, coronavirus disease 2019; PVA, patient-ventilator asynchrony; ARDS, acute respiratory distress syndrome; IMV, invasive mechanical ventilation.

METHODS

Study Design and Setting

The study was conducted in the First People's hospital of Jingmen. Clinical data and ventilator wave data were retrospectively collected. All ventilator parameters were collected as longitudinally in hourly basis using a ventilator information system (RespCare^{TM}, ZhiRuiSi Tech. Co., Ltd., Hangzhou, China). The impact of RM and positioning on lung compliance was explored in mixed linear model. The study was approved by the ethics committee of the First People's hospital of Jingmen (Approval number: 202002007) and the ethics committee of Sir Run Run Shaw hospital (20200407-32). Individual patient data were de-identified before analysis. Informed consent was waived as determined by the IRB due to retrospective nature of the study design.

Participants

All COVID-19 patients treated with IMV were included for analysis. COVID-19 was confirmed by one of the following criteria: (1) novel coronavirus nucleic acid was positive as confirmed by real time (RT)-PCT in respiratory or blood specimen; and (2) genetic sequencing showed highly homogenous sequence with the known novel coronavirus (16). For adults with COVID-19 and acute hypoxemic respiratory failure despite conventional oxygen therapy (<92%), we would start using high-flow nasal cannula (HFNC) or non-invasive ventilation (NIV). If the condition further deteriorated and the oxygenation saturation could not be maintained above 92% with HFNC or NIV, IMV would be started (17). Patients were excluded if (1) they were younger than 18 years old; (2) patients with do-not-resuscitate order and (3) with terminally ill disease; (4) patients with incomplete record of waveform data.

Variables

Demographic data including age and sex were collected as time-fixed data. Hospital mortality was obtained on discharge. Pulmonary functions including forced vital capacity (FVC), forced expiratory volume (FEV1), FEV1/FVC ratio, peak expiratory flow (PEF), Peak inspiratory flow (PIF), maximal inspiratory pressure (MIP), maximal expiratory pressure (MEP) were measured for hospital survivors.

Ventilator parameters including lung compliance, measured PEEP, plateau pressure, tidal volume, work of breathing (WOB), and peak flow rate were measured based on pressure and flow waveforms. Details of the measurement approaches are described in the ESM.

Interventions including RM, positioning, sedatives and neuromuscular blockades were recorded in our analysis. Date

and time of these interventions used to match to a period when ventilator parameters and lung mechanics were recorded. The body position was recorded as one of supine, right, left and prone positions at a specific time. Non-supine position was applied during daytime, and the specific positioning (prone, right or left) was determine at the discretion of the attending physician and respiratory therapist depending on the improvement in oxygenation. Prone positioning was applied for at least 10 h one day. RM could be accurately identified from ventilator waves as those with more than 30 cmH_2O sustained inflation maintained for at least 30 s, the upper limit pressure was 45 cmH_2O (18).

Identification of DT and IEE

We developed an interpretable deep learning approach to detect double triggering (DT) and ineffective inspiratory effort during expiration (IEE). Individual deep learning models were developed under all ventilation modes. Under each ventilation mode, two models were established for detecting DT and IEE. Each model uses the raw ventilator waveforms (airway pressure and flow) as input for a binary classification (PVA or non-PVA). It is also capable of explaining the classification by highlighting the segments that contributes mostly to the results. Datasets were annotated by a group of clinical professionals for training and validating the models based on our previously proposed

TABLE 1 | Clinical characteristics and lung mechanics of individual subject.

Variables	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7
Age (years)	57	57	66	81	68	68	54
Sex	Male	Female	Female	Female	Male	Female	Male
Hours from hospital admission to intubation	45	18	163	95	0	116	37
Comorbidities	None	None	Hypertension; diabetes; hepatitis	Hypertension; stroke	Hypertension; diabetes	None	None
Recruitment maneuver (counts)	4	1	0	0	0	0	0
Ejection fraction	58%	61%	58%	60%	50%	NA	NA
Pro-BNP (pg/ml)	521.3	377.1	125.7	1531.0	690.6	687.5	1313.0
CRP (MG/dl)	97.8	16.2	14.6	9.7	88.2	42.3	121.5
CK (U/L)	49.5	154.9	53.0	91.1	119.9	46.8	196.7
CK-MB (U/L)	7.6	18.2	9.6	6.3	9.6	8.2	13.4
LDH (IU/L)	508.5	401.6	453.2	499.4	494.5	482.0	562.8
Troponin T (ng/L)	16.11	16.29	5.50	39.09	38.37	18.88	361.9
Bacteriology (sample/pathogen)	Blood/ *Enterococcus faecium*	Sputum/ *Acinetobacter baumannii*	Negative	Blood/ *Enterococcus faecium*	Negative	Sputum/ *Stenotrophomonas maltophilia*	Sputum/ *Acinetobacter baumannii*
Chest CT involvement	Bilateral	Bilateral	Bilateral	Bilateral	Bilateral	Bilateral	Bilateral
Lesion pattern	Ground glass	Consolidation	Ground glass	Ground glass	Consolidation	Ground glass	Ground glass
Antiviral therapy	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Antibiotics	Yes	Yes	Yes	Yes	Yes	Yes	Yes
AI	6.35 (0.68, 21.89)	14.91 (4.62, 27.59)	5.78 (3.12, 33.97)	1.96 (0.97, 4.23)	6.42 (3.17, 14.26)	0.66 (0.23, 2.72)	21.92 (11.5, 42.38)
Lung compliance (cmH_2O)	12.15 (10.06, 14.31)	12.17 (10.72, 14.49)	12.45 (11.03, 14.22)	29.04 (23.65, 34.16)	11.24 (9.94, 12.92)	15.74 (14.06, 18.16)	23.2 (17.03, 29.96)
PEEP	8.07 (7.6, 9.82)	9.56 (9.04, 9.82)	5.26 (5.05, 7.03)	8.69 (4.69, 9.51)	7.22 (6.48, 7.86)	7.67 (6.11, 9.39)	7.26 (6.46, 7.66)
Plateau pressure (cmH_2O)	30.22 (27.95, 33.65)	30.87 (28.05, 34.02)	30.6 (28.35, 35.47)	24 (20.16, 26.01)	31.1 (30.03, 35.56)	32.53 (30.74, 33.84)	28.16 (23.99, 32.66)
Tidal volume (ml)	335.74 (249.1, 405.16)	276.19 (238.69, 329.52)	302.37 (280.24, 339.13)	477.07 (430.38, 523.45)	274.73 (258.55, 292.78)	454.52 (432.26, 474.59)	425.13 (371.11, 474.42)
Respiratory rate (/min)	26.35 (23.4, 31.94)	23.95 (20.09, 28.12)	26.73 (23.45, 28.91)	23.22 (19.86, 29.56)	22.97 (19.97, 27.37)	29.97 (27.92, 30.14)	26.07 (23.39, 28.67)
WOB (J/L)	0.69 (0.51, 0.95)	0.68 (0.6, 0.81)	0.74 (0.68, 0.82)	0.78 (0.67, 0.89)	0.6 (0.57, 0.71)	1.16 (1.05, 1.21)	0.97 (0.78, 1.08)
Peak flow rate (ml/min)	55.69 (47.21, 65.24)	43.35 (37.68, 55.54)	42.22 (40.57, 56.21)	50.25 (44.3, 57.07)	74.94 (68.96, 79.37)	57.83 (56.26, 60.28)	69.95 (58.66, 78.49)
Mortality	Died	Died	Alive	Alive	Died	Died	Alive

AI, asynchrony index; WOB, work of breathing; PEEP, positive end expiratory pressure; LDH, Lactate Dehydrogenase; CRP, C-reactive protein; BNP, B-type natriuretic peptide; CK, Creatine kinase.

TABLE 2 | Longitudinal variables compared between survivors and non-survivors.

Variables	Total (n = 2,778 h)	Alive (n = 1,160 h)	Died (n = 1,618 h)	p
Neuromuscular blockades, *n* (%)	81 (3)	23 (2)	58 (4)	0.018
Sedative, *n* (%)	305 (11)	156 (13)	149 (9)	<0.001
Recruitment maneuver, *n* (%)	5 (0)	0 (0)	5 (0)	0.079
Asynchrony Index (%), Median (IQR)	4.95 (1.69, 18.93)	4.84 (2.16, 16.29)	5.07 (1.11, 20.04)	0.007
Compliance, Median (IQR)	12.28 (10.4, 15.22)	15.41 (12.26, 20.85)	11.19 (9.77, 12.8)	<0.001
Position, *n* (%)				<0.001
Prone	499 (18)	248 (21)	251 (16)	
Right	404 (15)	145 (12)	259 (16)	
Left	453 (16)	225 (19)	228 (14)	
Supine	1422 (51)	542 (47)	880 (54)	
Plateau pressure (cmH_2O), Median (IQR)	28.44 (24.9, 32.17)	26.46 (23, 29.23)	30.26 (27.3, 33.1)	<0.001
PEEP (cmH_2O), Median (IQR)	7.92 (6.87, 9.64)	7.15 (5.14, 8.6)	9.09 (7.68, 9.79)	<0.001
Tidal volume (ml), Median (IQR)	356.44 (274.71, 445.04)	422.95 (343.21, 487.8)	298.98 (249.24, 390.7)	<0.001
Respiratory rate (/min), Median (IQR)	25.65 (21.91, 29.04)	25.49 (22.03, 28.91)	25.78 (21.83, 29.54)	0.052
WOB, Median (IQR)	0.75 (0.61, 0.94)	0.81 (0.69, 0.94)	0.7 (0.58, 0.94)	<0.001
Peak flow rate (ml/min), Median (IQR)	54.43 (44.08, 64.08)	53.8 (43.48, 63.31)	54.69 (44.75, 64.4)	0.654
DT (/h), Median (IQR)	29 (7, 65)	35 (14, 65)	24 (3, 65.75)	<0.001
IEE (/h), Median (IQR)	29 (4, 153)	21 (5, 100.25)	41 (3, 176)	0.025

WOB, work of breathing; PEEP, positive end expiratory pressure; DT, delayed triggering; IEE, ineffective effort during expiration; IQR, interquartile range.

approach (19). The accuracy reached above 95% for both types of PVA in all the ventilation modes. Asynchrony index (AI) was calculated as the number of asynchronous events divided by the number of ventilator cycles and wasted efforts (14). Details of the algorithm development is described in the Electronic **Supplemental Material**.

Statistical Analysis

Ventilator parameters were described for each individual patient by median and interquartile range (IQR) (20). Temporal trends of ventilator parameters were visualized with scatter plots and described with Locally Weighted Scatterplot Smoothing (LOWESS) curves (21). These curves were drawn for each individual patient and survivors and non-survivors were denoted with different colors.

Risk factors for IEE and DT were explored with mixed negative binomial regression models, which was a generalization of the Poisson regression allowing for the conditional variance exceeds the conditional mean (22). Random-effects was allowed for intercepts to account for between-subject variance. Predictors of IEE and DT included compliance, plateau pressure, PEEP, TV, respiratory rate, peak flow rate, WOB, sedatives, and neuromuscular blockades. We reported relative risk (RR) for the risk estimate associated with a unit change of these predictors. Risk factors for AI was explored with mixed linear effect model because the response variable AI was in linear scale. We reported coefficient and 95% confidence interval (CI) to represent how AI increased with a unit change in predictors. Factors that can influence lung compliance was explored with a mixed-effects linear model. Factors including age, sex, RM, PEEP, AI, and body position were included in the model. All statistical analyses were performed with RStudio (Version 1.1.463). A two-tailed $p < 0.05$ were considered as statistical significance.

RESULTS

Participants and Descriptive Analysis

A total of 7 patients with full record of ventilator waveforms were included for analysis. There was no excluded patient due to predefined exclusion criteria. Four patients died and three survived to hospital discharge (**Table 1**). A total of 3,923,450 respiratory cycles in 2,778 h were analyzed (average: 24 cycles/min) for the seven patients. Demographics and ventilator parameters were described in **Table 1**. Due to the limited number of patients, statistical inference was not performed for patient level data. Survivors showed significantly higher lung compliance [15.41 (12.26, 20.85) vs. 11.19 (9.77, 12.8) ml/cmH_2O; $p < 0.001$], lower PEEP [7.15 (5.14, 8.6) vs. 9.09 (7.68, 9.79) cmH_2O; $p < 0.001$] and plateau pressure [26.46 (23, 29.23) vs. 30.26 (27.3, 33.1); $p < 0.001$] than non-survivors. Survivors were more likely to adopt prone position than non-survivors (21 vs. 16%; $p < 0.001$). All RM was performed in non-survivors. More neuromuscular blockades were used in non-survivors (**Table 2**). Patients were on supine position in 1,422 h (51%), followed by prone positioning (499 h, 18%), left positioning (453 h, 16%), and right positioning (404 h, 15%). Survivors showed increasing compliance over time, whereas non-survivors showed persistently low compliance (**Figure 1A**). Plateau pressure, PEEP and tidal volume are shown in **Figures 1B–D**. WOB and respiratory rate did not show difference between survivors and non-survivors in temporal pattern (**Figures 1E,F**). Temporal trends of PVA were not different between survivors and non-survivors (**Figure 2**).

Factors Associated With PVA

Risk factors for PVA (IEE and DT) were investigated in the mixed negative binomial regression models. Higher plateau pressure (RR: 0.945; 95% CI: 0.934–0.956; $p < 0.001$) and respiratory rate (RR: 0.963; 95% CI: 0.951–0.976; $p < 0.001$) was associated with less IEE. However, greater tidal volume and WOB were associated with more IEE. In contrast to IEE, higher respiratory rate was associated with increased risk of DT (RR: 1.066; 95% CI: 1.054–1.078; $p < 0.001$). Higher plateau pressure (Coefficient: −0.90; 95% CI: −1.02 to −0.78) and neuromuscular blockades (Coefficient: −6.54; 95% CI: −9.92 to −3.16) were associated with lower AI. Sedatives had no significant impact on PVAs (**Table 3**).

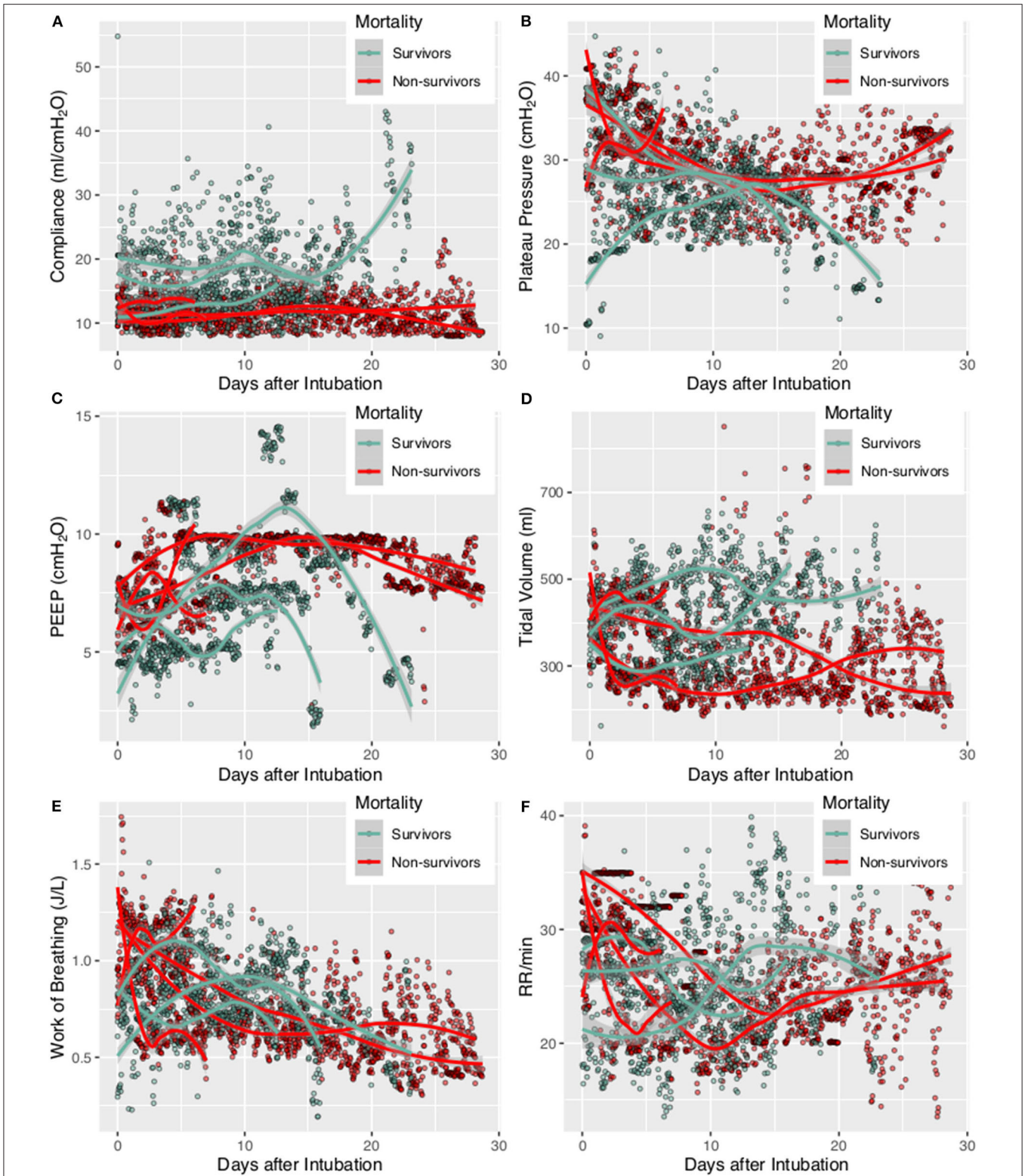


FIGURE 1 | Trajectories of lung mechanics in survivors and non-survivors. The scatter points were smoothed with Locally Weighted Scatterplot Smoothing method. **(A)** Lung compliance was higher in survivors than in non-survivors. **(B)** Plateau pressure of non-survivors followed a U-shaped curve. **(C)** PEEP followed a N-shaped curve with high values during the middle period. **(D)** Tidal volume was higher in survivors, probably due to better lung compliance. **(E)** Consistently decreasing work of breathing was observed in non-survivors. **(F)** Respiratory rate was higher at the beginning, declined rapidly during treatment and reach a nadir at 10–15 days. The respiratory rate was stabilized thereafter at 20–25 per min.

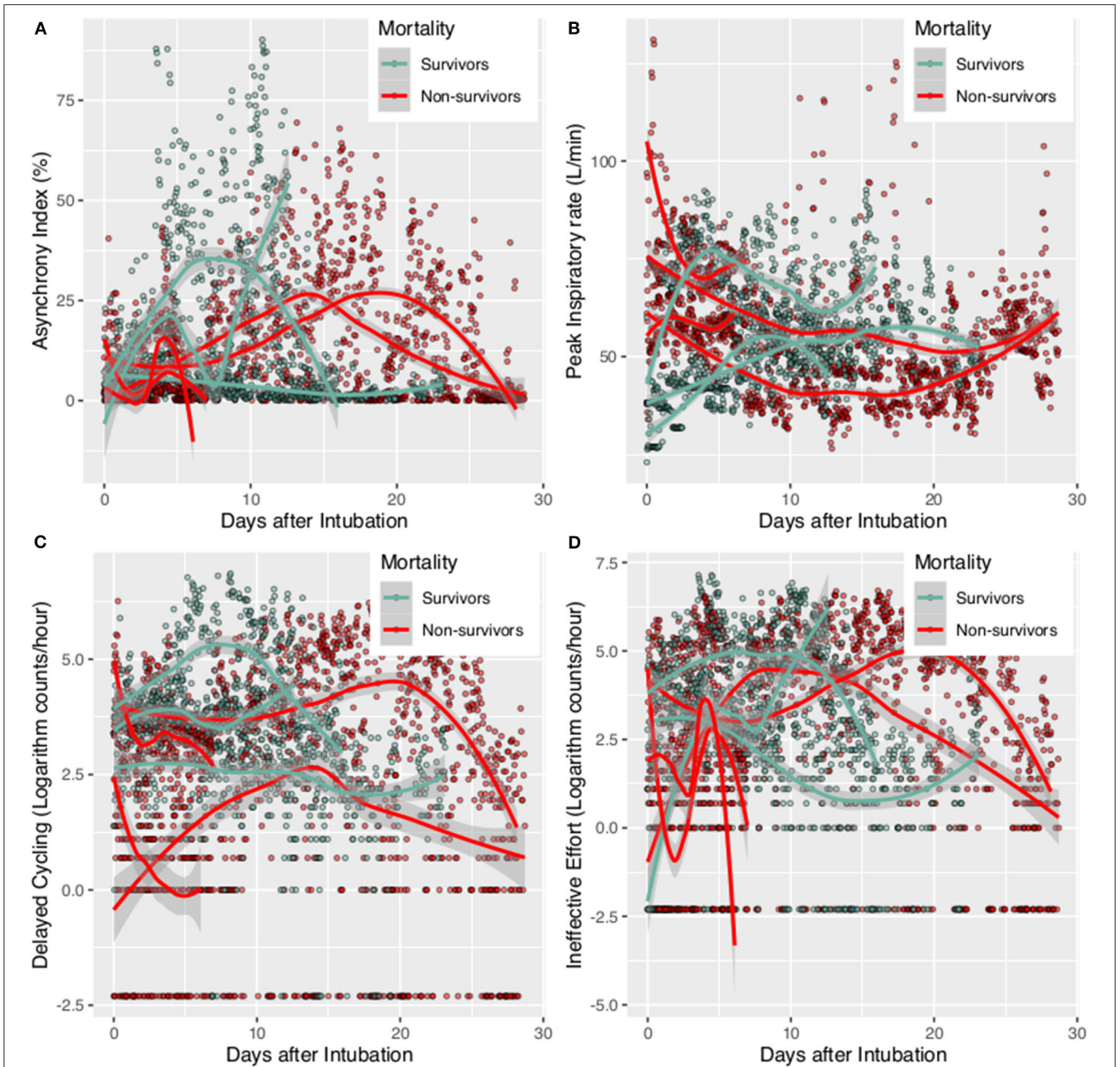


FIGURE 2 | Patient-ventilator Asynchrony during mechanical ventilation for survivors and non-survivors. The scatter points were smoothed with Locally Weighted Scatterplot Smoothing method. **(A)** Asynchrony index trajectory for individual patients; **(B)** Peak inspiratory rate for individual patient; **(C)** delayed cycling for individual patient; **(D)** Ineffective Effort for individual patient.

Lung Compliance

In multivariable mixed-effects linear model, we found two variables were significantly associated with lung compliance. Each 1 cmH_2O increase in PEEP was associated 0.27 ml/cmH_2O decrease in lung compliance (95% CI: −0.36 to −0.18; $p < 0.001$). As compared with supine positioning, prone positioning was associated with 2.31 ml/cmH_2O (95% CI: 1.75–2.86; $p < 0.001$) increase in lung compliance. Right (coefficient: 1.63; 95% CI: 1.08–2.19 ml/cmH_2O; $p < 0.001$) and left (coefficient: 0.63; 95% CI: 0.20–1.06 ml/cmH_2O; $p = 0.004$) positioning were both associated with improve lung compliance (**Table 4**).

Spirometry Test for Survivors

Spirometry tests were performed in survivors at day 8, 11, and 13 after extubation. It showed that FVC was consistently decreased for the three measurements. FEV1/FVC was decreased in patient 3 (0.73 at day 8 and 0.707 at day 11); but was preserved in

TABLE 3 | Mixed negative binomial regression model exploring risk factors for asynchrony.

Variables	RR for IEE (95% CI)	*p*	RR for DT (95% CI)	*p*	Coefficient for AI (95% CI)	*p*
Compliance	0.991 (0.977, 1.005)	0.199	1.005 (0.994, 1.017)	0.345	0.17 (0.03, 0.31)	0.016
Plateau pressure	0.945 (0.934, 0.956)	<0.001	0.962 (0.953, 0.972)	<0.001	−0.90 (−1.02, −0.78)	<0.001
PEEP	1.018 (0.982, 1.056)	0.337	1.122 (1.091, 1.154)	<0.001	1.56 (1.23, 1.88)	<0.001
Tidal volume	1.003 (1.002, 1.004)	<0.001	1.003 (1.002, 1.003)	<0.001	0.02 (0.01, 0.02)	<0.001
Respiratory rate	0.963 (0.951, 0.976)	<0.001	1.066 (1.054, 1.078)	<0.001	−0.12 (−0.24, −0.00)	0.049
Peak flow rate	0.996 (0.991, 1.001)	0.082	0.998 (0.993, 1.002)	0.226	−0.07 (−0.11, −0.02)	0.008
WOB	4.066 (2.954, 5.595)	<0.001	2.562 (2.007, 3.272)	<0.001	8.52 (5.80, 11.25)	<0.001
Neuromuscular blockades	0.5 (0.355, 0.704)	<0.001	0.576 (0.434, 0.764)	<0.001	−6.54 (−9.92, −3.16)	<0.001
Sedatives	0.959 (0.797, 1.153)	0.657	1.072 (0.923, 1.246)	0.362	1.33 (−0.49, 3.14)	0.152

WOB, work of breathing; PEEP, positive end expiratory pressure; DT, delayed triggering; IEE, ineffective effort during expiration; RR, relative risk.

TABLE 4 | Mixed linear model exploring factors associated with compliance.

Variables	Coefficient (95% CI)	*p*
Sex (Female as reference)	5.14 (−9.11, 19.39)	0.334
Recruitment	0.40 (−3.14, 3.94)	0.825
PEEP	−0.27 (−0.36, −0.18)	<0.001
Age (with each year increase)	0.22 (−0.32, 0.76)	0.291
Days from admission to intubation	0.02 (−0.03, 0.07)	0.291
Asynchrony Index (with each 1% increase)	0.01 (−0.00, 0.02)	0.113
Body position (supine as reference)		
Prone	2.31 (1.75, 2.86)	<0.001
Right	1.63 (1.08, 2.19)	<0.001
Left	0.63 (0.20, 1.06)	0.004

PEEP, positive end expiratory pressure; CI, confidence interval.

TABLE 5 | Spirometry tests for survivors after extubation.

	Patient 3 (Day 8)	Patient 3 (Day 11)	Patient 7 (Day 14)
FVC/predicted FVC	1,223/2,419	1,152/2,419	1,078/3,777
FEV1/predicted FEV1	896/1,849	884/1,849	865/1,789
FEV1/FVC	0.73	0.707	0.850
PEF/predicted PEF	103/350	171/350	65/544
PIF	70	107	58
MIP/predicted MIP	43/71	27/71	15/113
MEP/predicted MEP	43/130	42/130	22/212

FVC, forced vital capacity; FEV1, forced expiratory volume; FEV1/FVC ratio; PEF, peak expiratory flow; PIF, peak inspiratory flow; MIP, maximal inspiratory pressure; MEP, maximal expiratory pressure.

patient 7. PEF, MIP, and MEP were all decreased for the three measurements (**Table 5**).

DISCUSSION

The study integrated high-granularity ventilator waveform data with clinical variables to describe the temporal change of lung mechanics of critically ill patients with COVID-19. At the time of intubation, the lung compliance was similar in survivors and non-survivors; but the survivors showed gradually improved compliance. Prone positioning is effective in improve lung compliance. Two types of PVA, IEE, and DT, were identified with deep learning algorithm. Higher plateau pressure and use of muscular relaxant were associated with lower risk of PVAs. Spirometry tests showed that pulmonary functions were significantly compromised after recovery from COVID-19 induced ARDS. Long-term follow up for the change of pulmonary functions would be relevant.

Although the lung compliance was similar at the time of intubation, survivors showed gradual improvement in lung compliance, while non-survivors showed persistently low lung compliance. This is consistent with other studies that lung compliance was an independent predictor of mortality (5, 23, 24). An important finding in our study was that RM was not effective in improving lung compliance, which is in contrast to findings from general ARDS patients. Although the effect of RM on mortality was conflicting in general ARDS, it has been consistently reported to be able to improve lung compliance (25–27). For example, Kung and colleagues observed that the respiratory system compliance was significantly higher in the RM group from day 1 to 7 (25). There is evidence that direct/pulmonary ARDS is more responsive to RM than indirect/extrapulmonary ARDS. While only 21% patients with lower percentage of recruitable lung were caused by pulmonary ARDS, 51% patients with higher percentage of recruitable lung caused by pulmonary ARDS ($p = 0.01$) (28). Thus, COVID-19 induced ARDS is pulmonary ARDS but is less responsive to RM as shown in our study. The second reasons may be due to the fact that we only employed sustained inflation RM. Since there are many types of RM, it is largely unknown whether other types of RM can be effective in improve lung compliance in COVID-19 patients. Finally, the ARDS in COVID-19 may be due to viral, bacterial, or any kind of lung insults. Thus, the RM should not be able to demonstrate the benefits in this group of patients.

PVAs are commonly observed in patients with IMV, especially those with protective ventilation strategy. Our study observed that AI was 4.95% (IQR: 1.69–18.93) in overall observed hours. Non-survivors had more AI than survivors, indicating AI is a risk factor for mortality, which was consistent with other studies (29).

Ventilator parameters can have differing effects on different PVA types. For example, while higher respiratory rate was associated with lower risk of IEE, it was associated with higher risk of DT. Use of neuromuscular blockades was associated with lower risk of both IEE and DT. However, we did not observe significant effect of sedatives on AI. Other studies have shown that Propofol or other sedatives can reduce AI (13, 30). It is not surprising to observe that neuromuscular blockades are associated with significantly reduced risk of PVAs.

Post-extubation pulmonary function has never been reported for COVID-19 patients. Our results indicated that pulmonary functions can be significantly compromised in a short period. The FVC is reduced to one third of the predicted value. Other pulmonary function parameters, such as PEF and MIP were also reduced by one third of the predicted value. Boucher and coworkers observed that the pulmonary function can be significantly compromised in pediatric ARDS in short follow-up period (31). In adult patients with general ARDS, the FVC can recover to 3.34 ± 0.77 and 3.78 ± 1.11 L at 1 and 6 months follow up (32), which is significantly higher than that in our study. However, since we did not obtain the long term follow up data, it is largely unknown whether COVID-19 can have long-term effect on pulmonary functions.

Several limitations should be acknowledged in the study. First, the sample size was limited, which prohibited patient-level analysis. The effect of prone-positioning on mortality outcome could not be analyzed with sufficient statistical power. Thus, further large-scale studies are needed to validate our findings. However, our data is rich with high-granularity waveform data, which allows for patient-hour analysis for epidemiological analysis. Second, we only developed deep learning algorithms for identifying two types of PVA. There are other types of PVAs, such as reverse triggering and short/long cycling. However, these analyses are not applicable in pressure-controlled ventilation and pleural pressure is required for reverse triggering (33). Third, the impact of sedative on PVAs were estimated without the dosing of sedatives. We only recorded the use of sedatives as a binary variable. Such treatment would lose some information but is easy to interpret because different sedatives imposes challenge to standardize the dose. Finally, the pulmonary function was measured in a short period of time; long-term follow up data may provide important information for critically ill COVID-19 patients.

CONCLUSIONS

In conclusion, the study for the first time described full trajectory of lung mechanics of patients with COVID-19. The result showed that prone positioning was associated with improved compliance; higher plateau pressure and use of neuromuscular blockades were associated with lower risk of AI. RM was not associated with improvement on compliance.

DATA AVAILABILITY STATEMENT

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

ETHICS STATEMENT

The study was approved by the ethics committee of the First People's Hospital of Jingmen (Approval number: 202002007) and the ethics committee of Sir Run Run Shaw hospital (20200407-32). Informed consent was waived as determined by the IRB due to retrospective nature of the study design.

AUTHOR CONTRIBUTIONS

HG, QP, and ZZ analyzed and interpreted the results and drafted the manuscript. JZ, CY, and LZ handled the FINNAKI data. All authors took part in designing the study, revised the manuscript critically for important intellectual content, read, and approved the final manuscript.

FUNDING

This study was supported by the National Natural Science Foundation of China (Grant 31870938), Zhejiang Provincial Key Laboratory of Communication Networks and Applications and Zhejiang Provincial Natural Science Foundation of China (Grant No. LY19H010005).

ACKNOWLEDGMENTS

The authors would like thank Biyun Lai and Jie Pan from College of Information Engineering, Zhejiang University of Technology for preparing the data, ZhiRuiSi Tech. Co., Ltd. for donating the RespCareTM system and Mr. Bing Li from ZhiRuiSi for deploying the system on the scene to help the current research work.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2020.00541/full#supplementary-material

REFERENCES

1. Guan W-J, Ni Z-Y, Hu Y, Liang W-H, Ou C-Q, He J-X, et al. Clinical characteristics of coronavirus disease 2019 in China. *N Engl J Med.* (2020) 382:1708–20. doi: 10.1056/NEJMoa2002032
2. Ki M, Task Force for 2019-nCoV. Epidemiologic characteristics of early cases with 2019 novel coronavirus (2019-nCoV) disease in Republic of Korea. *Epidemiol Health.* (2020) 42:e2020007. doi: 10.4178/epih.e2020007
3. Yang X, Yu Y, Xu J, Shu H, Xia J, Liu H, et al. Clinical course and outcomes of critically ill patients with SARS-CoV-2 pneumonia in Wuhan, China: a single-centered, retrospective, observational study. *Lancet Respir Med.* (2020) 8:475–81. doi: 10.1016/S2213-2600(20)30079-5

4. Pensier J, de Jong A, Hajjej Z, Molinari N, Carr J, Belafia F, et al. Effect of lung recruitment maneuver on oxygenation, physiological parameters and mortality in acute respiratory distress syndrome patients: a systematic review and meta-analysis. *Intensive Care Med.* (2019) 45:1691–702. doi: 10.1007/s00134-019-05821-9
5. Morales-Quinteros L, Schultz MJ, Bringué J, Calfee CS, Camprubí M, Cremer OL, et al. Estimated dead space fraction and the ventilatory ratio are associated with mortality in early ARDS. *Ann Intensive Care.* (2019) 9:128. doi: 10.1186/s13613-019-0601-0
6. Ramírez II, Adasme RS, Arellano DH, Rocha ARM, Andrade FMD, Núñez-Silveira J, et al. Identifying and managing patient-ventilator asynchrony: an international survey. *Med Intens.* (2019). doi: 10.1016/j.medin.2019.09.004. [Epub ahead of print].
7. Bassuoni AS, Elgebaly AS, Eldabaa AA, Elhafz AAA. Patient-ventilator asynchrony during daily interruption of sedation versus no sedation protocol. *Anesth Essays Res.* (2012) 6:151–6. doi: 10.4103/0259-1162.108296
8. Blanch L, Villagra A, Sales B, Montanyà J, Lucangelo U, Luján M, et al. Asynchronies during mechanical ventilation are associated with mortality. *Intensive Care Med.* (2015) 41:633–41. doi: 10.1007/s00134-015-3692-6
9. Vaporidi K, Babalis D, Chytas A, Lilitsis E, Kondili E, Amargianitakis V, et al. Clusters of ineffective efforts during mechanical ventilation: impact on outcome. *Intensive Care Med.* (2017) 43:184–91. doi: 10.1007/s00134-016-4593-z
10. Gattinoni L, Coppola S, Cressoni M, Busana M, Rossi S, Chiumello D. Covid-19 does not lead to a "typical" acute respiratory distress syndrome. *Am J Respir Crit Care Med.* (2020). doi: 10.1164/rccm.202003-0817LE. [Epub ahead of print].
11. See KC, Sahagun J, Taculod J. Defining patient-ventilator asynchrony severity according to recurrence. *Intensive Care Med.* (2020) 32:1515. doi: 10.1007/s00134-020-05974-y
12. De Haro C, Ochagavia A, López-Aguilar J, Fernandez-Gonzalo S, Navarra-Ventura G, Magrans R, et al. Patient-ventilator asynchronies during mechanical ventilation: current knowledge and research priorities. *Intensive Care Med Exp.* (2019) 7:43–4. doi: 10.1186/s40635-019-0234-5
13. Conti G, Ranieri VM, Costa R, Garratt C, Wighton A, Spinazzola G, et al. Effects of dexmedetomidine and propofol on patient-ventilator interaction in difficult-to-wean, mechanically ventilated patients: a prospective, open-label, randomised, multicentre study. *Crit Care.* (2016) 20:206–8. doi: 10.1186/s13054-016-1386-2
14. Sousa ML de A, Magrans R, Hayashi FK, Blanch L, Kacmarek RM, Ferreira JC. Predictors of asynchronies during assisted ventilation and its impact on clinical outcomes: the EPISYNC cohort study. *J Crit Care.* (2020) 57:30–5. doi: 10.1016/j.jcrc.2020.01.023
15. Zhang Z, Reinikainen J, Adeleke KA, Pieterse ME, Groothuis-Oudshoorn CGM. Time-varying covariates and coefficients in Cox regression models. *Ann Transl Med.* (2018) 6:121. doi: 10.21037/atm.2018.02.12
16. Jin Y-H, Cai L, Cheng Z-S, Cheng H, Deng T, Fan Y-P, et al. A rapid advice guideline for the diagnosis and treatment of 2019 novel coronavirus (2019-nCoV) infected pneumonia (standard version). *Mil Med Res.* (2020) 7:4–23. doi: 10.1186/s40779-020-0233-6
17. Alhazzani W, Møller MH, Arabi YM, Loeb M, Gong MN, Fan E, et al. Surviving sepsis campaign: guidelines on the management of critically ill adults with coronavirus disease 2019 (COVID-19). *Intensive Care Med.* (2020) 44:1691–34. doi: 10.1007/s00134-020-06022-5
18. Arnal J-M, Paquet J, Wysocki M, Demory D, Donati S, Granier I, et al. Optimal duration of a sustained inflation recruitment maneuver in ARDS patients. *Intensive Care Med.* (2011) 37:1588–94. doi: 10.1007/s00134-011-2323-0
19. Zhang L, Mao K, Duan K, Fang S, Lu Y, Gong Q, et al. Detection of patient-ventilator asynchrony from mechanical ventilation waveforms using a two-layer long short-term memory neural network. *Comput Biol Med.* (2020) 120:103721. doi: 10.1016/j.compbiomed.2020.103721
20. Zhang Z. Univariate description and bivariate statistical inference: the first step delving into data. *Ann Transl Med.* (2016) 4:91. doi: 10.21037/atm.2016.02.11
21. Cleveland WS. LOWESS: a program for smoothing scatterplots by robust locally weighted regression. *Am. Stat.* (1981) 35:54. doi: 10.2307/2683591
22. Kondo Y, Zhao Y, Petkau J. A flexible mixed-effect negative binomial regression model for detecting unusual increases in MRI lesion counts in individual multiple sclerosis patients. *Stat Med.* (2015) 34:2165–80. doi: 10.1002/sim.6484
23. Kim J-S, Kim Y-J, Kim M, Ryoo SM, Sohn CH, Ahn S, et al. Impact of lung compliance on neurological outcome in patients with acute respiratory distress syndrome following out-of-hospital cardiac arrest. *J Clin Med.* (2020) 9:527. doi: 10.3390/jcm9020527
24. Bellani G, Grassi A, Sosio S, Gatti S, Kavanagh BP, Pesenti A, et al. Driving pressure is associated with outcome during assisted ventilation in acute respiratory distress syndrome. *Anesthesiology.* (2019) 131:594–604. doi: 10.1097/ALN.0000000000002846
25. Kung S-C, Hung Y-L, Chen W-L, Wang C-M, Chang H-C, Liu W-L. Effects of stepwise lung recruitment maneuvers in patients with early acute respiratory distress syndrome: a prospective, randomized, controlled trial. *J Clin Med.* (2019) 8:231. doi: 10.3390/jcm8020231
26. Goligher EC, Hodgson CL, Adhikari NKJ, Meade MO, Wunsch H, Uleryk E, et al. Lung recruitment maneuvers for adult patients with acute respiratory distress syndrome. A systematic review and meta-analysis. *Ann Am Thorac Soc.* (2017) 14:S304–11. doi: 10.1513/AnnalsATS.201704-340OT
27. Spadaro S, Mauri T, Böhm SH, Scaramuzzo G, Turrini C, Waldmann AD, et al. Variation of poorly ventilated lung units (silent spaces) measured by electrical impedance tomography to dynamically assess recruitment. *Crit Care.* (2018) 22:26–9. doi: 10.1186/s13054-017-1931-7
28. Gattinoni L, Caironi P, Cressoni M, Chiumello D, Ranieri VM, Quintel M, et al. Lung recruitment in patients with the acute respiratory distress syndrome. *N Engl J Med.* (2006) 354:1775–86. doi: 10.1056/NEJMoa052052
29. Bruni A, Garofalo E, Pelaia C, Messina A, Cammarota G, Murabito P, et al. Patient-ventilator asynchrony in adult critically ill patients. *Miner Anesthesiol.* (2019) 85:676–88. doi: 10.23736/S0375-9393.19.13436-0
30. Jakob SM, Ruokonen E, Grounds RM, Sarapohja T, Garratt C, Pocock SJ, et al. Dexmedetomidine vs midazolam or propofol for sedation during prolonged mechanical ventilation: two randomized controlled trials. *JAMA.* (2012) 307:1151–60. doi: 10.1001/jama.2012.304
31. Boucher V, Mathy C, Lacroix J, Émériaud G, Jouvet P, Tse SM. Post-discharge respiratory outcomes of children with acute respiratory distress syndrome. *Pediatr Pulmonol.* (2020) 55:468–73. doi: 10.1002/ppul.24581
32. Toufen C Jr., De Santis Santiago RR, Hirota AS, Carvalho ARS, Gomes S, Amato MBP, et al. Driving pressure and long-term outcomes in moderate/severe acute respiratory distress syndrome. *Ann Intensive Care.* (2018) 8:119. doi: 10.1186/s13613-018-0469-4
33. Akoumianaki E, Lyazidi A, Rey N, Matamis D, Perez-Martinez N, Giraud R, et al. Mechanical ventilation-induced reverse-triggered breaths: a frequently unrecognized form of neuromechanical coupling. *Chest.* (2013) 143:927–38. doi: 10.1378/chest.12-1817

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Medicine

BRIEF RESEARCH REPORT
published: 21 August 2020
doi: 10.3389/fmed.2020.00471

COVID-19 Infection Among Healthcare Workers: Serological Findings Supporting Routine Testing

Ariel D. Stock[1†], Edward R. Bader[1,2†], Phillip Cezayirli[1], Julio Inocencio[1], Samantha A. Chalmers[3], Reza Yassari[1], Vijay Yanamadala[1] and Emad Eskandar[1,2]*

[1] Montefiore Medical Center Department of Neurological Surgery, Bronx, NY, United States, [2] Albert Einstein College of Medicine Department of Neuroscience, Bronx, NY, United States, [3] Albert Einstein College of Medicine Department of Microbiology and Immunology, Bronx, NY, United States

A growing body of evidence demonstrates that asymptomatic and pre-symptomatic transmission of SARS-CoV-2 is a major contributor to the COVID-19 pandemic. Frontline healthcare workers in COVID-19 hotspots have faced numerous challenges, including shortages of personal protective equipment (PPE) and difficulties acquiring clinical testing. The magnitude of the exposure of healthcare workers and the potential for asymptomatic transmission makes it critical to understand the incidence of infection in this population. To determine the prevalence of asymptomatic SARS-CoV-2 infection amongst healthcare workers, we studied frontline staff working in the Montefiore Health System in New York City. All participants were asymptomatic at the time of testing and were tested by RT-qPCR and for anti-SARS-CoV-2 antibodies. The medical, occupational, and COVID-19 exposure histories of participants were recorded via questionnaires. Of the 98 asymptomatic healthcare workers tested, 19 (19.4%) tested positive by RT-qPCR and/or ELISA. Within this group, four (4.1%) were RT-qPCR positive, and four (4.1%) were PCR and IgG positive. Notably, an additional 11 (11.2%) individuals were IgG positive without a positive PCR. Two PCR positive individuals subsequently developed COVID-19 symptoms, while all others remained asymptomatic at 2-week follow-up. These results indicate that there is considerable asymptomatic infection with SARS-CoV-2 within the healthcare workforce, despite current mitigation policies. Furthermore, presuming that asymptomatic staff are not carrying SARS-CoV-2 is inconsistent with our results, and this could result in amplified transmission within healthcare settings. Consequently, aggressive testing regiments, such as testing frontline healthcare workers on a regular, multi-modal basis, may be required to prevent further spread within the workforce and to patients.

Keywords: COVID-19, SARS-CoV-2, healthcare worker, asymptomatic infection, coronavirus, asymptomatic infection carriers

OPEN ACCESS

Edited by:
Abdallah Samy,
University of Kansas, United States

Reviewed by:
Ian George Barr,
The University of Melbourne, Australia
Ahmed Mostafa,
National Research Centre, Egypt

***Correspondence:**
Ariel D. Stock
adstock@montefiore.org

†These authors share first authorship

Specialty section:
This article was submitted to Infectious Diseases Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: *13 May 2020*
Accepted: *13 July 2020*
Published: *21 August 2020*

Citation:
Stock AD, Bader ER, Cezayirli P, Inocencio J, Chalmers SA, Yassari R, Yanamadala V and Eskandar E (2020) COVID-19 Infection Among Healthcare Workers: Serological Findings Supporting Routine Testing. Front. Med. 7:471. doi: 10.3389/fmed.2020.00471

INTRODUCTION

Throughout the progression of the COVID-19 pandemic, healthcare workers (HCWs) have experienced high levels of exposure to SARS-CoV-2, with the risk of infection rising with each time point of exposure (1, 2). HCWs are at greatest risk of SARS-CoV-2 infection, representing a large percentage of new infections. This, in part, has related to challenges in acquiring adequate personal

protective equipment (3), resulting in a great deal of anxiety and distress amongst providers due to concern for self-infection with COVID-19 and family exposure (4). An important element in the discussion of community and healthcare-worker infection relates to asymptomatic and pre-symptomatic transmission of COVID-19, which may occur in up to 30% of individuals (5–7). New York City has been more severely affected then most (8), with widespread community infection, including a significant high-acuity disease burden (9). Attempts to address this concern are currently in their infancy, with widespread rollout of PCR-based and serological assessment in their early phases (10). Results of a recent New York State pilot study, which randomly tested 15,000 residents for serological evidence of SARS-CoV-2 exposure, found over 10% seroconversion statewide, with nearly 25% seropositivity in New York City (11). Importantly, we do not yet know the significance of seropositivity against SARS-CoV-2, particularly since most serological studies have been done in patients with a history of severe disease, and relative titers in asymptomatic carriers may not indicate immunity from transmission or infection (12).

Given the elevated risk of COVID-19 infection among HCWs and the consequent distress and concern from potential asymptomatic infection and transmission, we endeavored to address the rate of asymptomatic or possibly resolved infection among HCWs. We proceeded to test a cohort of clinicians at our institution for COVID-19 infection, including those working in COVID-19 intensive care units, specialty service physicians, and ambulatory staff. We evaluated both current infection via RT-qPCR sampling for SARS-CoV-2 and serology for the presence of anti-SARS-CoV-2-IgG antibodies. Beyond assessing the rate of active and resolving infections within our clinicians, internal testing would allow us to help prevent further spread of COVID-19 by serving as a screening tool, keeping any infected, asymptomatic HCWs quarantined pending disease presentation. Finally, the ability to reassure our HCWs that they are not infected and identify HCWs who may have silently recovered from COVID-19 is important for attenuating worker anxiety.

METHODS

Study Design and Oversight

This cross-sectional study was approved by the Albert Einstein College of Medicine Institutional Review Board, with all subjects providing written informed consent. The goal of this study was to identify both asymptomatic HCW carriers of the SARS-CoV-2 virus, as well as those that may be immune to the virus, as denoted by serum IgG anti-SARS-CoV-2-nucleocapsid (IgG-anti-n) antibodies. These results would then assist in determining the safe deployment of staff within the hospital system to meet the demands of the COVID-19 healthcare crisis as well as provide an assessment of the rate of clinician infection in a COVID-19 hotspot.

Study Participants/Demographics

Adult clinicians working within the Montefiore Health System, Bronx, New York City, active during the COVID-19 pandemic, were recruited to participate in the study (testing conducted between 04/04–20/2020). Three positive control samples (initially testing positive by RT-qPCR between 3/23–4/5/2020 and at least 2 weeks prior to serum sampling for serology) were included as well. The goal was to sample clinicians with varying degrees of hospital exposure to COVID-19 patients who were not exhibiting typical symptoms of COVID-19 (including fever, cough, and shortness of breath) at the time of participation. Exclusion criteria included an age over 65, as the risk of infection during testing outweighed the benefits, and individuals with any signs or symptoms typical of COVID-19. Each participant completed a survey pertaining to the current COVID-19 pandemic, exposure, workplace histories, recent history of symptoms attributable to COVID-19 infection, and medical history (used to calculate the Charlson Comorbidity Index score). Statistical relationships between groups were calculated using the Pearson's Chi-squared test and Fisher's Exact test for categorical variables, the Mann-Whitney-U test for continuous variables, and the Kruskal Wallis H test for comparison of IgG titers.

SARS-CoV-2 RT-qPCR Testing

Participants underwent both nasopharyngeal and oropharyngeal swabbing concurrently, and samples were pooled. We collected swabs directly into the RNA-lysis buffer, and we then isolated RNA using a Zymo Research RNA MicroKit (Irvine, CA) according to manufacturer's recommendations. Each RNA sample was evaluated by spectrophotometry and then analyzed by RT-qPCR according to CDC-recommended protocols (13) for SARS-CoV-2 testing with slight modification, utilizing primers to the *nucleocapsid* gene (N1 and N2) and *RnaseP* (RP) as a control (IDTDNA, Coralville, IA). Commercially available plasmid controls were utilized for all primer sequences (IDTDNA). After validating accuracy on several positive controls and redundantly running the reaction on multiple samples, the reaction volume was scaled down from a 96-well-plate format to a 384-well-plate format, with samples run on the Applied Biosystems Via7 system and analyzed using the QuantStudio software package (Thermo Scientific, Waltham, MA).

ELISA for Anti-SARS-CoV-2-Nucleocapsid IgG

Blood was collected from each participant into serum separator tubes (BD, Franklin Lakes, NJ), allowed to coagulate at room temperature for 60 min, and then stored at 4°C until centrifugation. Serum was analyzed in duplicate using an anti-n IgG ELISA (Epitope Diagnostics Inc., San Diego, CA), according to manufacturer's recommendations with slight modification. Assay cut-off values per the protocol were determined as follows: the optical densities of the negative control samples (all of which between 0.19 and 0.22) were averaged and adjusted by addition of a constant (0.18). This resultant reference value was then multiplied by a correction factor of 1.1 (which represents the cutoff value); anything above this being positive and anything below being negative. In addition to the internal controls provided with the kit, we included three participants with a history of RT-qPCR-positive SARS-CoV-2 infection as positive controls.

TABLE 1 | Demographics and clinical characteristics of frontline healthcare providers tested for SARS-CoV-2.

Demographics and clinical variables	Total ($n = 98$)	Positive[a] ($n = 19$)	Negative ($n = 79$)	*P*-value[b]
Mean age (+/– SD)- yr.	37.6 (10.6)	38.8 (13.7)	37.4 (9.8)	0.815[1]
Sex—no. (%)				1.000[2]
Male	49 (50.0)	10 (52.6)	39 (49.4)	
Female	49 (50.0)	9 (47.4)	40 (50.6)	
Job Type—no. (%)				0.240[3]
Both inpatient and outpatient	86 (87.8)	15 (79.0)	71 (89.9)	
Exclusively outpatient	12 (12.3)	4 (21.5)	8 (10.1)	
Job Title—no. (%)				0.415[3]
Physician	62 (63.3)	12 (63.2)	50 (63.3)	
Physician's Assistant	15 (15.3)	4 (21.5)	11 (13.9)	
Nurse Practitioner	9 (9.2)	3 (15.8)	6 (7.6)	
Nurse	6 (6.1)	0	6 (7.6)	
Perfusionist	6 (6.1)	0	6 (7.6)	
SARS-CoV-2 Exposure Risk Index- no. (%)				0.292[3]
No known exposure	7 (7.1)	1 (5.3)	6 (7.6)	
Wearing full PPE	57 (58.2)	8 (42.1)	49 (62.0)	
Conventional droplet precautions	16 (16.3)	5 (26.3)	11 (13.9)	
No PPE	18 (18.4)	5 (26.3)	13 (16.5)	
Typical COVID-19 symptoms- no. (%)				0.052[2]
Absent	67 (68.4)	9 (47.3)	58 (73.4)	
Present	31 (31.6)	10 (52.6)	21 (26.6)	
Comorbidities—no. (%)				0.785[2]
None	68 (69.4)	14 (73.7)	54 (68.4)	
Asthma	10 (10.2)	2 (10.5)	8 (10.1)	
Hypertension	5 (5.1)	2 (10.5)	3 (3.8)	
Hyperlipidemia	5 (5.1)	0	5 (6.3)	
Malignancy	4 (4.1)	1 (5.3)	3 (3.8)	
Autoimmune disease	3 (3.1)	0	3 (3.8)	
Diabetes mellitus	2 (2.0)	1 (5.3)	1 (1.3)	
Inflammatory bowel disease	2 (2.0)	0	2 (2.5)	
Endocrine disorder	2 (2.0)	0	2 (2.5)	
Hematological disorder	2 (2.0)	0	2 (2.5)	
Charlson Comorbidity Index – no. (%)				0.205[3]
0	80 (81.6)	13 (68.4)	67 (84.0)	
1	10 (10.2)	4 (21.1)	6 (7.6)	
2	4 (4.1)	1 (5.3)	3 (3.8)	
3	1 (1.0)	0	1 (1.3)	
4	3 (3.1)	1 (5.3)	2 (2.5)	

[a]Defined as a positive swab PCR and/or positive serum IgG ELISA. [b]P-values calculated using [1]Mann-Whitney U test, Pearson's [2]Chi-squared test, or [3]Fisher's exact test.

TABLE 2 | IgG ELISA optical densities of frontline healthcare workers tested for SARS-CoV-2.

SARS-CoV-2 test results	Total ($N = 98$)	Mean IgG ELISA OD (+/– SEM)	*P*-value[a]
Result profile groups			<0.001[1]
PCR Positive—no. (%)	4 (4.1)	0.251 (0.032)	
PCR/IgG Positive—no. (%)	4 (4.1)	0.656 (0.055)	
IgG Positive—no. (%)	11 (11.2)	0.589 (0.081)	
PCR/IgG Negative—no. (%)	79 (80.6)	0.231 (0.008)	
IgG results by prior symptom profile			0.112[2]
No history of typical Covid symptoms—no. (%)	67 (68.4)	0.264 (0.016)	
Previous history of Covid symptoms—no. (%)	31 (31.6)	0.343 (0.043)	

[a]P-values calculated using [1]Kruskal-Wallis H test when comparing multiple groups, and [2]Mann-Whitney U test.

Performance of Clinically Administered SARS-CoV-2 Testing

To assess the performance of clinically administered testing, biostatistics were calculated by comparing hospital-administered RT-qPCR testing with the anti-n IgG ELISA testing we employed, using anti-n IgG ELISA as the reference standard for historical infection in this case. Only individuals whose clinically administered RT-qPCR test occurred ≥14 days before anti-n IgG ELISA testing were included to allow time for a detectable IgG antibody response to develop. Sensitivity, specificity, positive predictive value, negative predictive value, and accuracy were calculated alongside 95% confidence intervals.

RESULTS

Subject Characteristics

We evaluated 98 clinicians working in the Montefiore Health System who have been clinically active since the early part of the COVID-19 pandemic within New York City. Several work environments were represented, including COVID-19 medicine units, COVID-19 ICUs, the ED, specialty consultants, and those working in a purely ambulatory setting. These individuals had varying degrees of workplace exposure to COVID-19 patients, including invasive bedside procedures with COVID-19-positive patients, intraoperative exposure, as well as in routine care. Interestingly, overall exposure histories were not correlated with testing results ($p = 0.292$, **Table 1**). Additionally, a history of COVID-19-like illness was not correlated with optical densities on ELISA ($p = 0.112$, **Table 2**). Importantly, none of the subjects were symptomatic at the time of testing, though some were previously tested due to exposure and/or typical COVID-19 symptoms.

RT-qPCR for Active Infection

Nasopharyngeal and oropharyngeal swabs underwent RNA purification, and spectrophotometry revealed excellent RNA

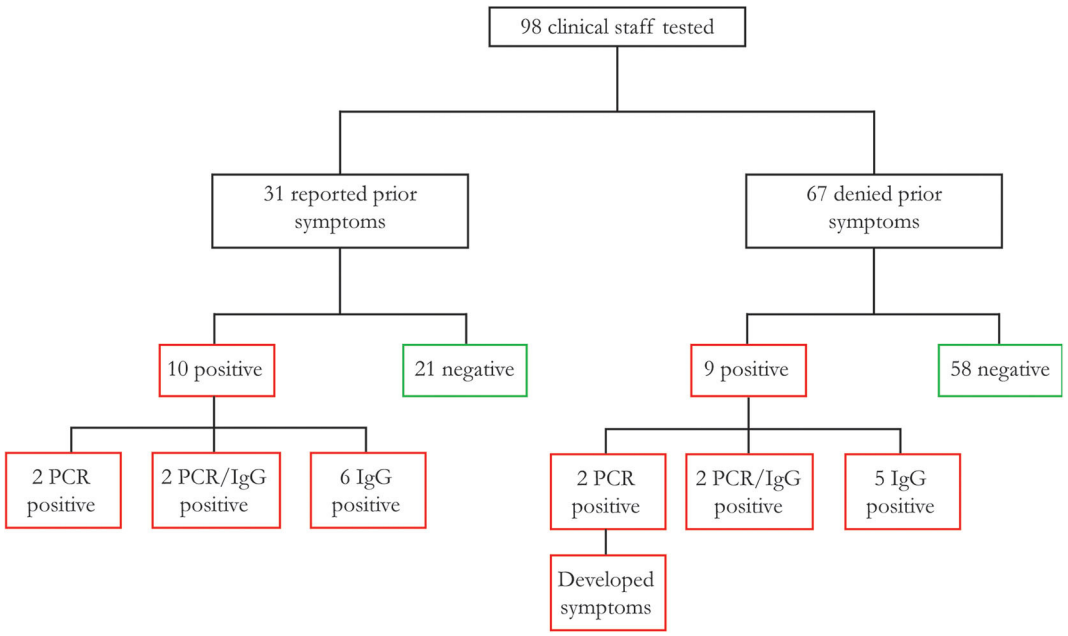


FIGURE 1 | Association between prior complaint of COVID-19 symptoms and SARS-CoV-2 infection of 98 healthcare worker participants, 31 reported typical COVID-19 symptoms prior to study participation, of whom 32.3% were found to have a history of SARS-CoV-2 infection (as defined by RT-qPCR or serology). There were an additional nine participants with no symptoms prior to study participation, of whom two subsequently developed symptoms (PCR positive), and with others asymptomatic as of this publication.

yields and quality. RT-qPCR was run with 10 ng RNA per reaction in triplicates for each primer (N1, N2, and RP). All samples demonstrated RP expression, indicating adequate RNA isolation from respiratory epithelium. Through viral RNA amplification, we identified a total of eight individuals who were SARS-CoV-2 positive (PCR positive, 8% of tested clinical staff, Ct values are the average of triplicates for each positive sample, standard deviations of all triplicates <1, subjects 1–4 and 8–11, **Figure 1**, **Table S1**), including four with a history of resolved symptoms and two who subsequently developed moderate symptoms. Two other individuals noted vague upper respiratory symptoms in retrospect but were otherwise asymptomatic.

Serum Anti-SARS-CoV-2-Nucleocapsid IgG

For serum evaluation, we focused on IgG for two reasons: it represents a more predictable and durable immune response than IgM and, once positive, should persist so for an extended period (14, 15). From a technical perspective, IgG is more readily and specifically assayable given its higher affinity for individual antigens (16). On ELISA, the positive control individuals, who had typical COVID-19 symptoms that resolved over 14 days prior to participation, had optical densities (ODs) above the positive threshold (0.560, 1.494, 1.166; PCR/IgG positive, subjects 5–7, **Table S1**), which is consistent with the literature on IgG responses during this pandemic (15). We further identified 15 individuals who met criteria for seropositivity (IgG positive, subjects 8–22, **Table S1**). Of the IgG positive group, four individuals were RT-qPCR positive (PCR/IgG positive, subjects 8–11) and 11 RT-qPCR negative (IgG positive, subjects 12–22). Interestingly, of the four PCR/IgG-positive individuals, two had a history of symptoms but were unable to secure testing due to limited availability. Among the 11 IgG-positive individuals, four had a history of symptoms with negative test results.

A total of 19 individuals had a history of clinically administered SARS-CoV-2 testing by RT-qPCR ≥14 days prior to participation in this study and anti-n IgG ELISA testing (**Table S1**). Of these 19, three tested positive on clinically administered testing; however, seven of these individuals tested positive by anti-n IgG ELISA in this study. When evaluating all those with a history of testing prior to this study, and assuming the validity of ELISA as the reference standard for history of infection (i.e., IgG positive = Infectious History), the sensitivity of clinically administered RT-qPCR based diagnostics for SARS-CoV-2 was only 42.86% (true positives/true positives + false negatives: 3/7 individuals), though importantly the specificity was 100% (true negatives/true negatives + false positives: 12/12 individuals) (**Table 3**). Subject #1 was characterized as a true negative, presumably having contracted SARS-CoV-2 in the interim.

DISCUSSION

There has been extensive discussion among healthcare providers, researchers, and policy makers about the role that asymptomatic, undiagnosed infections play in the spread of SARS-CoV-2 (7). Additionally, HCWs have found themselves inadequately supplied with personal protective equipment while caring for COVID-19 patients (3). Finally, the types of social distancing,

TABLE 3 | Sensitivity and specificity of clinically administered RT-qPCR testing prior to study participation (using anti-n IgG ELISA as the reference standard).

Statistic	Value (*n*/*n* individuals)	95% CI
Sensitivity	42.86% (3/7)	9.90–81.59%
Specificity	100% (12/12)	73.54–100.00%
Positive predictive value	100% (3/3)	
Negative predictive value	75.00% (12/16)	61.23–85.07%
Accuracy	78.95%	54.43–93.95%

*Sensitivity and specificity of clinically administered RT-qPCR testing for SARS-CoV-2 infection prior to participation in this study was calculated to include false negatives determined by current serology testing. Anti-n IgG ELISA was used as the reference standard. Subject #1 (**Table S1**) may represent a false negative or may have contracted the virus subsequent to clinical testing and was consequently characterized as a true negative.*

which, at the time of this writing, are having a successful impact on decreasing community SARS-CoV-2 spread, are not practically feasible within the healthcare work environment. Consequently, it is reasonable to expect an increased incidence of SARS-CoV-2 infection among healthcare workers. In this study, of the 98 asymptomatic healthcare workers tested, we identified 19 (19.4%) SARS-CoV-2-positive participants, as defined by PCR and/or serology.

Addressing the impact of SARS-CoV-2 among HCWs requires identifying those in the pre-symptomatic/asymptomatic phase as well as those who may have had the infection and may now be at an attenuated risk of infection or transmission. In a recent publication evaluating the SARS-CoV-2 transmission pattern in an early Washington State skilled nursing facility cluster, the authors highlighted the role that asymptomatic and/or pre-symptomatic transmission between residents certainly played in disease dissemination (7, 17). Furthermore, their findings support the inadequacy of relying on symptomatic presentation as the indicator for testing healthcare providers. The latter finding is consistent with our own in which more than 10% of asymptomatic HCWs presented with SARS-CoV-2 profiles consistent with either recent infection or seroconversion. Institutional and national testing limitations, meanwhile, represented a problem at the start of this pandemic, though routine screening of HCWs is recently available. Given the limited ability to test minimally symptomatic individuals during the early months of the pandemic, policymakers have largely suggested identifying those that have recovered, through serology, as an element of return to societal function in the future. The independent testing presented herein utilized both approaches, presenting important findings both regarding the infectious status of healthcare workers, as well as issues with PCR-based testing sensitivity, owing to fluctuating viral loads and sampling technique variability. Additionally, as the vast majority of testing has been validated in symptomatic individuals, the sensitivity of PCR in asymptomatic individuals, such as the ones studied here, remains uncertain.

We identified 19 of 98 clinicians (19.4%) that demonstrated either a new diagnosis (PCR positive) or a history of SARS-CoV-2 (IgG positive). These included four (4.1%) PCR-positive, four (4.1%) PCR/IgG-positive, and 11 (11.2%) PCR-negative/IgG-positive individuals. Of the 19 SARS-CoV-2 positive participants, 10 (10.2%) reported a prior history of COVID-19 symptoms, now presenting as two PCR-positive, two PCR/IgG-positive, and six IgG-positive individuals (**Figure 1**, **Table 1**). Of the nine participants without a prior history of COVID-19 symptoms, two were PCR-positive individuals that subsequently developed symptoms with an additional two PCR/IgG-positive and five IgG-positive participants that remained asymptomatic for 2 weeks after testing. Among all 16 (16.3%) participants with negative clinical testing for SARS-CoV-2 prior to study participation, four (4/16, 25%) were found to be IgG positive (suggesting prior false negative testing), and one (1/16, 6.3%) was PCR positive.

The IgG testing poses the primary limitation in this study or any of its kind at this time. While the assay has reportedly undergone validation with positive and negative controls, is accompanied by internal controls, and has demonstrated positivity with our own control participants, the novelty of available assays requires careful interpretation. For example, the recombinant nucleocapsid protein used in the chosen assays shares some degree of homology with other coronaviruses, including some, to which the studied population are routinely exposed (18, 19). Additionally, the cutoffs for positivity and negativity in the study, while reportedly validated by the supplier on positive control and pre-pandemic sera, leave an interval in between positive and negative results that are unclear (which we considered negative for the purposes of this study). Other assays, which use total viral lysate as a plated antigen, would pose similar challenges. Recent developments focused on a modified Spike protein, which appears to have improved *in-vitro* stability, as well as a more specific binding affinity for SARS-CoV-2, are underway as well (20). Most importantly, we have yet to determine whether seroconversion confers longstanding, seasonal, or limited immunity, making serology of limited, diagnostic utility at this time (12).

Despite inherent limitations in newly developed serological assays and their interpretation, RT-qPCR behaved as expected. A number of individuals were found to be persistently PCR positive, after an extended period of time from symptom onset, consistent with reports elsewhere (21–24). This feature of COVID-19 has the ancillary benefit of lending confidence to our IgG results, as there was concordance between testing results in nearly 40% of IgG positive individuals. The most significant of our findings, in line with the primary goal of this study, was *de novo* identification of eight asymptomatic individuals amongst clinicians that were PCR positive for SARS-CoV-2. This represents critical information in terms of staff management and deployment. Indeed, several participant HCWs, functioning in essential settings on the frontline of patient care, were pulled from active duty shortly prior to developing symptoms.

A better understanding of the dynamics of healthcare-worker infection with SARS-CoV-2 is essential in protecting this key element of the workforce as well as mitigating their role in nosocomial and community spread of COVID-19. While this study represents a limited snapshot, it identifies

several important findings. Foremost, healthcare workers may be carrying and, therefore, spreading SARS-CoV-2, without any signs or symptoms of disease. Additionally, prior negative testing by PCR does not preclude infection, which can be identified serologically. We applaud government efforts to scale-up serological and PCR-based testing programs, but we caution that an individual timepoint may not provide adequate mitigation of SARS-CoV-2 transmission by and between healthcare workers. In future studies, it will be interesting to evaluate HCWs over time, to determine the rate of infection in a group with known regular exposure. Collectively, our findings suggest that it is appropriate to regularly test all healthcare workers in high disease burden areas for SARS-CoV-2 by both PCR and serological assays, irrespective of ostensible exposure or symptom history.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Albert Einstein College of Medicine IRB. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AS conceived this study, executed most of the testing, and wrote much of this manuscript. EB performed data analysis, assisted with manuscript writing and data interpretation. PC assisted with data collection and analysis. JI assisted with sample collection and processing. SC assisted with study design and sample processing. RY assisted in data interpretation. VY helped design this study. EE was involved with the conception, design, and interpretation of this study. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

We would like to acknowledge the assistance of the Naomi Berezin, MD, and David Weithorn, MD, in the recruitment of participants. We would additionally like to dedicate this study to Dr. James Tait Goodrich, a leader within our department and world-renowned pediatric neurosurgeon, who was lost to COVID-19.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2020.00471/full#supplementary-material

REFERENCES

1. Alhazzani W, Moller MH, Arabi YM, Loeb M, Gong MN, Fan E, et al. Surviving sepsis campaign: guidelines on the management of critically ill adults with coronavirus disease 2019 (COVID-19). *Crit Care Med*. (2020) 48:e440–69. doi: 10.1097/CCM.0000000000004363
2. McMichael TM, Currie DW, Clark S, Pogosjans S, Kay M, Schwartz NG, et al. Epidemiology of Covid-19 in a long-term care facility in King County, Washington. *N Engl J Med*. (2020) 382:2005–11. doi: 10.1056/NEJMoa2005412
3. Ranney ML, Griffeth V, Jha AK. Critical supply shortages - the need for ventilators and personal protective equipment during the Covid-19 pandemic. *N Engl J Med*. (2020) 382:e41. doi: 10.1056/NEJMp2006141
4. Joseph B, Joseph M. The health of the healthcare workers. *Indian J Occup Environ Med*. (2016) 20:71–2. doi: 10.4103/0019-5278.197518
5. Mizumoto K, Kagaya K, Zarebski A, Chowell G. Estimating the asymptomatic proportion of coronavirus disease 2019 (COVID-19) cases on board the Diamond Princess cruise ship, Yokohama, Japan, 2020. *Euro Surveill*. (2020) 25:2000180. doi: 10.2807/1560-7917.ES.2020.25.10.2000180
6. Dugan A. For physicians and nurses on the front line, there's worry over bringing coronavirus home: 'I can't even go home and hug my kids anymore'. *Boston Globe* (2020, March 20).
7. Arons MM, Hatfield KM, Reddy SC, Kimball A, James A, Jacobs JR, et al. Presymptomatic SARS-CoV-2 infections and transmission in a skilled nursing facility. *N Engl J Med*. (2020) 382:2081–90. doi: 10.1056/NEJMoa2008457
8. Dong E, Du H, Gardner L. An interactive web-based dashboard to track COVID-19 in real time. *Lancet Infect Dis*. (2020) 20:533–4. doi: 10.1016/S1473-3099(20)30120-1
9. Richardson S, Hirsch JS, Narasimhan M, Crawford JM, McGinn T, Davidson KW, et al. Presenting characteristics, comorbidities, and outcomes among 5700 patients hospitalized with COVID-19 in the New York City area. *JAMA*. (2020) 323:2052–9. doi: 10.1001/jama.2020.6775
10. Deblasio B. *Mayor de Blasio Announces COVID-19 Antibody Testing for Healthcare Workers*. (2020). Available online at: https://www.nychealthandhospitals.org/pressrelease/mayor-de-blasio-announces-covid-19-antibody-testing-for-healthcare-workers/
11. Branch NYSE. *Video, Audio, Photos & Rush Transcript: Amid Ongoing COVID-19 Pandemic, Governor Cuomo Announces Results of Completed Antibody Testing Study of 15,000 People Show 12.3 Percent of Population has Covid-19 Antibodies*. (2020).
12. Altmann DM, Douek DC, Boyton RJ. What policy makers need to know about COVID-19 protective immunity. *Lancet*. (2020) 395:1527–9. doi: 10.1016/S0140-6736(20)30985-5
13. *CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel*. Document #: CDC-006-00005 (2020). Available online at: https://www.fda.gov/media/134922/download
14. Li Z, Yi Y, Luo X, Xiong N, Liu Y, Li S, et al. Development and clinical application of a rapid IgM-IgG combined antibody test for SARS-CoV-2 infection diagnosis. *J Med Virol*. (2020) 1–7. doi: 10.1002/jmv.25727
15. To KK, Tsang OT, Leung WS, Tam AR, Wu TC, Lung DC, et al. Temporal profiles of viral load in posterior oropharyngeal saliva samples and serum antibody responses during infection by SARS-CoV-2: an observational cohort study. *Lancet Infect Dis*. (2020) 20:565–74. doi: 10.1016/S1473-3099(20)30196-1
16. Makela O, Ruoslahti E, Seppala IJ. Affinity of IgM and IgG antibodies. *Immunochemistry*. (1970) 7:917–32. doi: 10.1016/0019-2791(70)90053-4
17. Chow EJ, Schwartz NG, Tobolowsky FA, Zacks RLT, Huntington-Frazier M, Reddy SC, et al. Symptom screening at illness onset of health care personnel with SARS-CoV-2 infection in King County, Washington. *JAMA*. (2020) 323:2087–9. doi: 10.1001/jama.2020.6637
18. Spaan W, Cavanagh D, Horzinek MC. Coronaviruses: structure and genome expression. *J Gen Virol*. (1988) 69(Pt. 12):2939–52. doi: 10.1099/0022-1317-69-12-2939
19. Grifoni A, Sidney J, Zhang Y, Scheuermann RH, Peters B, Sette A. A sequence homology and bioinformatic approach can predict candidate targets for

immune responses to SARS-CoV-2. *Cell Host Microbe.* (2020) 27:671–80 e2. doi: 10.1016/j.chom.2020.03.002
20. Amanat F, Stadlbauer D, Strohmeier S, Nguyen THO, Chromikova V, McMahon M, et al. A serological assay to detect SARS-CoV-2 seroconversion in humans. *Nat Med.* (2020) 26:1033–6. doi: 10.1038/s41591-020-0913-5
21. Colavita F, Lapa D, Carletti F, Lalle E, Bordi L, Marsella P, et al. SARS-CoV-2 isolation from ocular secretions of a patient with COVID-19 in Italy with prolonged viral RNA detection. *Ann Intern Med.* (2020) 173:M20-1176. doi: 10.7326/M20-1176
22. Wu Y, Guo C, Tang L, Hong Z, Zhou J, Dong X, et al. Prolonged presence of SARS-CoV-2 viral RNA in faecal samples. *Lancet Gastroenterol Hepatol.* (2020) 5:434–5. doi: 10.1016/S2468-1253(20)30083-2
23. Xing YH, Ni W, Wu Q, Li WJ, Li GJ, Wang WD, et al. Prolonged viral shedding in feces of pediatric patients with coronavirus disease (2019). *J Microbiol Immunol Infect.* (2020) 53:473–80. doi: 10.1016/j.jmii.2020.03.021
24. Xu K, Chen Y, Yuan J, Yi P, Ding C, Wu W, et al. Factors associated with prolonged viral RNA shedding in patients with COVID-19. *Clin Infect Dis.* (2020) 71:799–806. doi: 10.1093/cid/ciaa351

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Public Health

OPINION
published: 21 August 2020
doi: 10.3389/fpubh.2020.00394

Efficacy of Nationwide Curfew to Encounter Spread of COVID-19: A Case From Jordan

Moawiah Khatatbeh[*†]

Department of Basic Medical Sciences, Faculty of Medicine, Yarmouk University, Irbid, Jordan

Keywords: COVID-19, curfew, Jordan, lockdown, Middle East

INTRODUCTION

As of 20th June 2020, more than 8.6 million people are confirmed to have the novel COVID-19 in more than 200 countries (1). To stop the spread of COVID-19, countries have responded in varied ways. However, the response depended on several factors, of which, the number of cases and timing of interventions are the most crucial.

In Jordan, the first case of COVID-19 was discovered on 2nd March 2020 (2). The government started immediate interventions. Patients were immediately and completely isolated in specialized hospitals and quarantine was initiated as per official guideline to the medical professionals and using all personal protective equipment (3).

It has been reported in the literature that individuals with other co-morbidities are at a greater risk for catching COVID-19 infection (4). The prevalence of chronic non-communicable diseases in Jordan is considered high. For example, 38% of deaths is attributed to cardiovascular diseases (5). In addition, a recent study from Jordan revealed that the prevalence of diabetes in men and women aged ≥25 years was 32.4 and 18.1%, respectively (6). Therefore, the government started using all media channels including social media, TV, press advertisements, and smart mobile applications to raise the attention of people about the disease, its mode of transmission, and preventive measures.

JORDAN'S RESPONSE TO THE INCREASED NUMBER OF COVID-19 CASES

During the first 2 weeks, Jordan had only one confirmed case of COVID-19 before starting to diagnose new cases in the third week (from 14 to 20 March 2020), after which the country started implementing vital interventions to combat the spread of the disease. These interventions were empowered by the activation of the National Defense Law on 17th March 2020. This law stipulates that, "upon a decision and a Royal Decree, a National Defense Law shall be passed in case of emergency that would threaten the national security or public safety in all parts of the Kingdom or in a region due to war, disturbances, armed internal strife, public disasters or the spread of a pest or epidemic" (7). The activation of the law led to suspension of the studies at educational institutions, closure of borders, stopping prayers in places of worship, and all large gatherings were banned.

After 19 days of discovery of the 1st case in Jordan (21 March 2020), 15 new cases were confirmed heading a total of 98 cases in the country, which constituted a red flag sign. On that day, several additional interventions have been taken, of which implementing complete nationwide curfew (24/24 h) for 3 days—22–24 March—was of great efficiency (8). After these 3 days of nationwide lockdown (from 25 March until the moment of writing this report), the government has implemented several days of complete curfews over weekends, in addition to the daily partial lockdowns.

OPEN ACCESS

Edited by:
Zisis Kozlakidis, International Agency for Research on Cancer (IARC), France

Reviewed by:
Mohammad S. Alyahya, Jordan University of Science and Technology, Jordan
Pierre Vodosin, International Agency for Research on Cancer (IARC), France

****Correspondence:***
Moawiah Khatatbeh
moawia.m@yu.edu.jo

†ORCID:
Moawiah Khatatbeh
orcid.org/0000-0002-6299-6486

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Public Health

Received: *16 April 2020*
Accepted: *06 July 2020*
Published: *21 August 2020*

Citation:
Khatatbeh M (2020) Efficacy of Nationwide Curfew to Encounter Spread of COVID-19: A Case From Jordan. Front. Public Health 8:394. doi: 10.3389/fpubh.2020.00394

During the curfew time, no one was allowed to move except the medical and nursing staff, police, and the armed forces. The government announced that these nationwide curfews will enable epidemiological investigation teams to trace patients' contacts and test them. Moreover, the complete curfew promotes social distancing and minimizes the number of new infections.

IMPACT OF CURFEW MEASURES

As of 20th June 2020, the total number of cases of COVID-19 in the country was 1,008 (2). This denotes that the curfew measures were effective and efficient. However, to assess the effectiveness of the lockdown, a comparison between Jordan and other countries in the Middle East would be beneficial in terms of the total number of cases relative to date of confirming the first case and time of implementing lockdowns. The number of cases divided by the total number of population will be also considered to understand the magnitude of the disease in each country. The number of population for each country was figured from the world live population meter, and total number of cases was figured from Johns Hopkins University's Coronavirus Resource Center, both measures as of 20th June 2020.

Results in **Table 1** show the latest statistics of COVID-19 in 12 Middle Eastern countries. It is noteworthy to say that countries including Syria and Palestine were excluded from this analysis because of their limited ability to contain the disease due to economic and political reasons.

DISCUSSION

Results in **Table 1** show that Jordan has the lowest number of COVID-19 cases compared to other countries in the Middle East. Despite Jordan having the disease before some countries like Turkey and Bahrain and having the disease on the same day as Saudi Arabia, these countries had very high number of cases compared to Jordan.

The lowest ratio of cases to the number of population was seen in Jordan (11/100,000) compared to all other countries. Qatar, Bahrain, UAE, Saudi Arabia, Turkey, and Iran had 3,015, 1,388, 437, 425, 238, and 217/100,000 population, respectively. Interestingly, among all countries, Qatar and Bahrain had the largest number of cases proportionate to the number of population.

The crucial factors in minimizing the infection rate are numerous. These factors may include demographic characteristics, precautions taken, public commitment, firmness in implementing measures, public awareness of the disease, national vaccinations' programs, and many other factors. However, in case of COVID-19, timing of implementing the lockdown is immensely crucial. In Jordan, the implementation of the nationwide curfew was stated as one of the weapons used in the battle against COVID-19 (9, 10). The country has implemented strict nationwide curfew measures at early stages compared to other countries. This early start and the relative low number of cases enabled the epidemiological investigation teams to detect the primary and secondary contacts and test them. In fact, many of the detected cases in Jordan were from those contacts. It is worth noting that the diagnostic tests are available in Jordan as more than 400,000 tests were carried out until the moment of preparing this report (2).

It seems that this was crucial in decreasing the infection rate. The effectiveness of the curfew was enhanced by the closure of all entry borders including the airport and the compulsory 14-days quarantine for all individuals arriving to Jordan within 3 days preceding closing the borders. Similarly, Oman has initiated curfew when they had only 20 cases. However, they have now a huge number of cases compared to Jordan. This likely refers to less strictly adopted curfew measures. All other countries included in this analysis implemented curfew measures at later

TABLE 1 | Number of cases of COVID-19 in 12 countries in the Middle East as of 20th June 2020.

Country	Date of first confirmed case	Number of cases at curfew point	Current total number of cases*	Number of cases/100,000 population**	Ratio of number of cases/100,000 population countries vs Jordan
Jordan	02 Mar 2020	98	1,008	11	-
Iran	19 Feb 2020	11,364	200,262	238	21.6
Turkey	11 Mar 2020	47,029	185,245	217	19.7
Saudi Arabia	02 Mar 2020	511	150,292	425	38.6
UAE	28 Jan 2020	611	44,145	437	39.7
Qatar	29 Feb 2020	562	85,462	3,015	274
Bahrain	16 Mar 2020	211	20,916	1,388	126.1
Kuwait	26 Feb 2020	176	38,678	782	71.1
Oman	25 Feb 2020	20	28,566	422	38.3
Lebanon	21 Feb 2020	368	1,510	19	1.7
Egypt	14 Feb 2020	363	52,211	51	4.6
Iraq	15 Mar 2020	124	27,368	64	5.8

**Figured from https://coronavirus.jhu.edu/map.html as of 20/06/2020.*

***Figured from https://countrymeters.info/en/World#population_clock as of 20/06/2020.*

stages and when they had larger number of cases compared to Jordan, which may explain their current statistics.

Disease statistics from Jordan reveal that interventions implemented, and precautions taken, especially the strict nationwide curfew, were successful in preventing the spread of the disease (9, 10). Despite the high prevalence rates of chronic diseases in the Jordanian population that put these vulnerable groups at higher risk for catching COVID-19 infection, the preventive measures and precautions taken by the country were effective in decreasing its spread. Among all countries in the Middle East, Jordan is in principle better positioned to respond to the outbreak of COVID-19 (11, 12). The effectiveness of the interventions is manifested by having the lowest number of cases in the Middle East and the country started documenting only few cases daily after 3 weeks of initiating the curfew. Nevertheless, implementing strict and frequent nationwide lockdowns has its price of impacting the economy due to the increased unemployment rate and losses in the gross domestic product especially in a country with limited resources, like Jordan, despite the latest World Bank classification of Jordan as an upper middle-income country (13). These adverse effects of the lockdown may have an impact on public mental health as a recent review reported that subsyndromal mental health problems are a common response to COVID-19 pandemic (14). The good news is that Jordan started relieving lockdown measures due to the controlled and comfortable epidemiological situation.

AUTHOR'S NOTE

Covid-19 has invaded more than 200 countries around the world. Countries have responded in varied ways to combat its spread. Western and developed countries are extremely suffering from huge cases numbers. However, in the Middle East, Jordan has implemented strict measures in fighting against the disease. Initiating nationwide curfew in the country, parallel with other interventions, has been effective in decreasing infection rate in Jordan. This effectiveness is manifested by having the lowest number of cases among Middle Eastern countries. Such interventions are important to be viewed and recognized by other countries, specially the developing ones, to fight against such pandemics.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

FUNDING

The publication of this paper was supported by the Deanship of Scientific Research and Graduate Studies at Yarmouk University.

REFERENCES

1. Johns Hopkins University Center. *COVID-19 Dashboard by the Center for Systems Science and Engineering (CSSE) at Johns Hopkins*. (2020). Available online at: https://coronavirus.jhu.edu/map.html (accessed on June 20, 2020).
2. Jordan's Ministry of health, Webpage for Coronavirus. Available online at: https://corona.moh.gov.jo/ar (accessed on July 4, 2020).
3. Suleiman A, Bsisu I, Guzu H, Santarisi A, Alsatari M, Abbad A, et al. Preparedness of frontline doctors in jordan healthcare facilities to COVID-19 outbreak. *Int J Environ Res Public Health*. (2020) 17:3181. doi: 10.3390/ijerph17093181
4. Huang R, Zhu L, Xue L, Liu L, Yan X, Wang, et al. Clinical findings of patients with coronavirus disease 2019 in Jiangsu province, China: a retrospective, multi-center study. *PLoS Negl Trop Dis*. (2020) 14:e0008280. doi: 10.1371/journal.pntd.0008280
5. Mokdad A. Global non-communicable disease prevention: building on success by addressing an emerging health need in developing countries. *J Health Special*. (2016) 4:92. doi: 10.4103/1658-600X.179820
6. Ajlouni K, Batieha A, Jaddou H, Khader Y, Abdo N, El-Khateeb M, et al. Time trends in diabetes mellitus in Jordan between 1994 and 2017. *Diabetic Med*. (2019) 36:1176–82. doi: 10.1111/dme.13894
7. What is the 'National Defense Law' that Jordan will likely pass to fight corona? Available online at: Roya news. 2020. Retrieved from: https://en.royanews.tv/news/20270/What-is-the--National-Defense-Law--that-Jordan-will-likely-pass-to-fight-corona? (accessed April 15, 2020).
8. Prime Ministry of Jordan. *Official Reports*. A List of Defense Law Orders (2020). Available online at: http://www.pm.gov.jo/upload/files/Order-Defense-2.pdf (accessed July 4, 2020).
9. Al-Tammemi AaB. The battle against COVID-19 in Jordan: an early overview of the jordanian experience. *Front Public Health*. (2020) 8:188. doi: 10.3389/fpubh.2020.00188
10. Alqutob R, Al Nsour M, Tarawneh MR, Ajlouni M, Khader Y, Aqel I, et al. COVID-19 crisis in Jordan: Response, scenarios, strategies, and recommendations. *JMIR Public Health Surveillance*. (2020) 6. doi: 10.2196/preprints.19332
11. Alkhawaldeh A. COVID-19: simultaneous surveillance studies and case series, Jordan as a case study. *Preprints*. (2020). doi: 10.20944/preprints202005.0071.v1
12. Aljazeera. *How Jordan is Flattening its COVID-19 Curve*. (2020). Available online at: https://www.aljazeera.com/news/2020/04/jordan-flattening-covid-19-curve-200422112212466.html (accessed on April 22, 2020).
13. The World Bank. *Jordan Profile*. (2020). Available online at: https://data.worldbank.org/country/JO (accessed on June 22, 2020).
14. Rajkumar RP. COVID-19 and mental health: a review of the existing literature. *Asian J Psychiatry*. (2020) 52:102066. doi: 10.1016/j.ajp.2020.102066

Conflict of Interest: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Public Health

OPINION
published: 25 August 2020
doi: 10.3389/fpubh.2020.00417

Ozone Therapy as a Possible Option in COVID-19 Management

Alessandra Gavazza[1], *Andrea Marchegiani*[1]*, *Giacomo Rossi*[1], *Marianno Franzini*[2,3], *Andrea Spaterna*[1], *Sara Mangiaterra*[1] *and Matteo Cerquetella*[1]

[1] School of Biosciences and Veterinary Medicine, University of Camerino, Matelica, Italy, [2] Oxygen-Ozone Therapy Scientific Society (SIOOT), Gorle, Italy, [3] Director of Comunian Clinic, Gorle, Italy

Keywords: COVID-19, ozone, public health, SARS-CoV-2, therapy

The rapid and pandemic outbreak of SARS-CoV-2 causing COVID-19 recognizes in the containment of the infection and in its therapeutic management the two most addressed and challenging topics. Recent guidelines suggest that person-to-person transmission (droplets and aerosol) are the main transmission routes and that, although less likely, also contact with surfaces and objects on which the virus is present can represent a risk (1, 2). With regard to treatment, many clinical trials are ongoing worldwide (3), but no specific antiviral treatment is unanimously recognized leaving to supportive care and symptoms management the most recommended approach (2, 4).

Ozone has extensively been studied in medicine and currently applied at different possible concentrations in various disciplines such as dentistry, dermatology, acute and chronic infectious diseases, and pneumology (5, 6). Chemically it is formed by a triatomic dynamically unstable molecule of oxygen that in gaseous form has a half-life of about 1 h at room temperature, rapidly reverting to oxygen (5). Regarding ozone-related risks, as environmental pollutant it has been shown to reduce maximal transpulmonary pressure, increases respiratory rate and decreased tidal volume as well as significantly increases mean airway resistance and specific airway resistance possibly contributing to increased Influenza A infection (6). Furthermore, it has been shown that the lipid peroxidation operated by high concentration of ozone at the alveolar level can cause strong structural alterations of the surfactant, in a dose and time dependent manner. Strong fusion of lamellar bodies (LBs), associated to the appearance of increasing concentrations of densely coiled LB-like shapes in the alveolar lavage, are resulting ultrastructural changes in type II alveolocites (7). At the same time, it occurs also a strong reduction of organized tubular myelin structures. This is likely due to the fact that medium-high concentration of ozone induce alveolar lesions as consequence of phospholipid peroxidation, causing time-dependent alterations in the organization of stored, and secreted surfactant membranes (8); as a result, administration of gaseous ozone must be avoided.

For medical purposes, ozone can be administered parenterally with minimal side effects, beside the only exception of not being injected intravenously as a gas because of the risk of embolism (5). As a powerful oxidant, when ozone comes into contact with blood or other body fluids, it releases reactive oxygen species (ROS), and lipid oxidation products (LOPs) both of which are responsible for the biological results (5). The main form of ROS is hydrogen peroxide (H_2O_2) which is easily transferred from plasma into the cells. When H_2O_2 abruptly appears above the threshold medical concentration in the cytoplasm of cells it represents the triggering stimulus for the possibly simultaneous activation of different biochemical pathways in erythrocytes, leukocytes and platelets in addition to other numerous biological effects, such as antimicrobial, immunostimulant, and antioxidant ones. H_2O_2 is then suddenly inactivated into water by the high concentration of glutathione (GSH), catalase (CAT), and glutathione peroxidase (GSH-Px) enzymatic systems, reducing its harmful potential (5). Although the exact mechanism of action of ozone is far to be fully elucidated, it has been characterized to have different biological properties. For example, it

OPEN ACCESS

Edited by:
Zisis Kozlakidis, International Agency for Research on Cancer (IARC), France

Reviewed by:
Atin Adhikari, Georgia Southern University, United States
Leopoldo Spadea, Sapienza University of Rome, Italy

***Correspondence:**
Andrea Marchegiani
andrea.marchegiani@unicam.it

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Public Health

Received: *06 April 2020*
Accepted: *13 July 2020*
Published: *25 August 2020*

Citation:
Gavazza A, Marchegiani A, Rossi G, Franzini M, Spaterna A, Mangiaterra S and Cerquetella M (2020) Ozone Therapy as a Possible Option in COVID-19 Management. Front. Public Health 8:417. doi: 10.3389/fpubh.2020.00417

has been showed to facilitate wound healing by promoting the release of oxygen, platelet-derived growth factor and transforming grow factor β (9). Ozone is also regarded as capable to activate the immune system increasing the production of interferon and interleukin-2 and decreasing tumor necrosis factor (TNF) levels (6). In addition to this, ozone stimulates both the red blood cell glycolysis rate leading to an increased amount of oxygen released to the tissues and the Krebs cycle resulting in an increased production of ATP. It also reduces significantly NADH concentration and helps to oxidize cytochrome C, thus stimulating oxygen metabolism (6), as well as it shows anti-inflammatory and possible cytoprotective action interacting with NF-KB and Nrf2 transcription agents (10, 11). The paradox that ozone exerts an antioxidant response (known as oxidative preconditioning) capable of reversing a chronic oxidative stress is related to the stimulation of production free radical scavengers and cell-wall protectors such as glutathione peroxidase, catalase, and superoxide dismutase (5, 12).

Through the oxidation of double bonds, ozone possesses the unique ability to inactivate biological contaminants, including viruses. Ozone disrupts the integrity of the bacterial cell walls causing their lysis and death (5, 13), and is able to effectively control spore germination of various dermatophytes (14, 15). Data obtained throughout years of research suggest that ozone inactivation of viruses occurs primarily in by lipid and protein peroxidation (16). Lipid peroxidation is initiated by different ROS, including H_2O_2. Through oxidation of the unsaturation along the hydrocarbon chain of fatty acid component of phospholipid membrane it causes severe structural and functional damage to the lipid bilayer of the plasma membrane (17). On the other hand, protein peroxidation is due either to interaction of protein with ROS or by interaction with secondary byproducts of oxidative stress; both of them cause irreversible oxidative changes that inhibit normal cellular mechanisms. These include loss of aggregation and proteolysis control, changes in enzyme-substrate binding activities, and modifications in immunogenicity (18). Protein peroxidation particularly seems to play a key role in the inactivation of non-enveloped viruses, such as adenovirus, poliovirus and other enteroviruses (19, 20). Murray and coworkers (21) demonstrated few years ago the efficacy of ozone against a variety of simple and complex viruses, including enveloped, non-enveloped, DNA, and RNA ones. Vesicular stomatitis Indiana virus (VSIV), adenovirus type-2 (HAdV-2), and selected strains of herpes simplex virus type-1 (HHV-1), vaccinia virus (VACV), influenza A virus (FLUAV) pools were exposed *in vitro* to a minimal amount of ozone (from 800 to 1,500 parts per million by volume), and it was effective in inactivating all these viruses. More in detail, enveloped viruses such as VSIV, HHV-1, VACV, and FLUAV showed great sensitivity to ozone while the non-enveloped HAdV-2 was more but not completely resistant to ozone. The results of the study suggest a direct and irreversible damage and destruction of the lipid viral envelope and protein capsid confirming the ability of ozone as a tool for the control of some viruses (21). Ozone therapy has recently been suggested as a possible economic and easily available further option for Sars-CoV-2 (22) thanks to its immunomodulatory, anti-inflammatory and biocide action and to the nitric oxide associated and dependent antiplatelet effect (23, 24). About the relationship between ozone and Sars-CoV-2 is also worth noting the "triangle" existing among human angiotensin-converting enzyme 2 (ACE2), that both is a receptor facilitating virus entry and, as fundamental component of renin-angiotensin system, also protects from acute lung injury, and Nrf2 pathway modulation, influencing ACE2 activity and being in turn influenced by ozone (10, 11, 25–27). Interestingly, the virus has also been found in substrates other than respiratory secretions, such as fecal swabs and blood (4), suggesting a possible interaction with the virus in case ozone is in the blood. Recently, the Italian "Istituto Superiore di Sanità" (National Institute of Health) answering to Prof. Franzini, member of "Scientific Society of Oxygen Ozone Therapy" Directive Board, recognized that oxygen-ozone therapy, after Ethical Committee approval and under patient informed consent, could represent a possible option (28). Remarkably, in this regard, two recent reports of the "Scientific Society of Oxygen Ozone Therapy," referring to patients affected by COVID-19 undergoing immediately after hospitalization, in addition to standard therapy, also to autohemotherapy with ozonated blood, furnished very encouraging results (29, 30). Moreover, also other reports hypothesizing the use of ozone in COVID-19 are being progressively undertaken and published (31, 32).

Gas concentration, route of administration, safety, stage of the disease in which administer it, patients' selection, contraindications, concomitant administration of antioxidants, etc., are some of the aspects that need to be further addressed with regard to its eventual use in COVD-19 patients, but in the authors opinion ozone therapy is an option that could deserve to be explored while waiting for specific treatments and for a vaccine.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

REFERENCES

1. CDC - How Coronavirus Spreads. Available online at: https://www.cdc.gov/coronavirus/2019-ncov/prevent-getting-sick/how-covid-spreads.html (accessed March 31, 2020).
2. Adhikari SP, Meng S, Wu YJ, Mao YP, Ye RX, Wang QZ, et al. Epidemiology, causes, clinical manifestation and diagnosis, prevention and control of coronavirus disease (COVID-19) during the early outbreak period: a scoping review. *Infect Dis Poverty*. (2020) 9:29. doi: 10.1186/s40249-020-00646-x
3. Li H, Zhou Y, Zhang M, Wang H, Zhao Q, Liu J. Updated approaches against SARS-CoV-2. *Antimicrob Agents Chemother*. (2020) 9:382–5. doi: 10.1128/AAC.00483-20
4. Guo Y-R, Cao Q-D, Hong Z-S, Tan Y-Y, Chen S-D, Jin H-J, et al. The origin, transmission and clinical therapies on coronavirus disease 2019

(COVID-19) outbreak – an update on the status. *Mil Med Res.* (2020) 7:1–10. doi: 10.1186/s40779-020-00240-0
5. Bocci VA. Scientific and medical aspects of ozone therapy. *State Art Arch Med Res.* (2006) 37:425–35. doi: 10.1016/j.arcmed.2005.08.006
6. Elvis AM, Ekta JS. Ozone therapy: a clinical review. *J Nat Sci Biol Med.* (2011) 2:66–70. doi: 10.4103/0976-9668.82319
7. Glew RH, Basu A, Shelley SA, Paterson JF, Diven WF, Montgomery MR, et al. Sequential changes of lamellar body hydrolases during ozone-induced alveolar injury and repair. *Am J Pathol.* (1989) 134:1143–50.
8. Balis JU, Paterson JF, Lundh JM, Haller EM, Shelley SA, Montgomery MR. Ozone stress initiates acute perturbations of secreted surfactant membranes. *Am J Pathol.* (1991) 138:847–57.
9. Valacchi G, Fortino V, Bocci V. The dual action of ozone on the skin. *Br J Dermatol.* (2005) 153:1096–100. doi: 10.1111/j.1365-2133.2005.06939.x
10. Martínez-Sánchez G, Schwartz A, Donna VD. Potential cytoprotective activity of ozone therapy in SARS-CoV-2/COVID-19. *Antioxidants (Basel).* (2020) 9:389. doi: 10.3390/antiox9050389
11. Valdenassi L, Franzini M, Ricevuti G, Rinaldi L, Galoforo AC, Tirelli U. Potential mechanisms by which the oxygen-ozone (O2-O3) therapy could contribute to the treatment against the coronavirus COVID-19. *Eur Rev Med Pharmacol Sci.* (2020) 24:4059–61. doi: 10.26355/eurrev_202004_20976
12. Larini A, Bianchi L, Bocci V. The ozone tolerance: I) Enhancement of antioxidant enzymes is ozone dose-dependent in Jurkat cells. *Free Radic Res.* (2003) 37:1163–8. doi: 10.1080/10715760310001604170
13. Sechi LA, Lezcano I, Nunez N, Espim M, Dupre I, Pinna A, et al. Antibacterial activity of ozonized sunflower oil (Oleozon). *J Appl Microbiol.* (2001) 90:279–84. doi: 10.1046/j.1365-2672.2001.01235.x
14. Wentworth P, McDunn JE, Wentworth AD, Takeuchi C, Nieva J, Jones T, et al. Evidence for antibody-catalyzed ozone formation in bacterial killing and inflammation. *Science.* (2002) 298:2195–9. doi: 10.1126/science.1077642
15. Ouf SA, Moussa TA, Abd-Elmegeed AM, Eltahlawy SR. Anti-fungal potential of ozone against some dermatophytes. *Brazilian J Microbiol.* (2016) 47:697–702. doi: 10.1016/j.bjm.2016.04.014
16. Dianzani MU. Lipid peroxidation and cancer. *Crit Rev Oncol Hematol.* (1993) 15:125–47. doi: 10.1016/1040-8428(93)90052-6
17. Esterbauer H, Schaur RJ, Zollner H. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radic Biol Med.* (1991) 11:81–128. doi: 10.1016/0891-5849(91)90192-6
18. Shacter E. Quantification and significance of protein oxidation in biological samples. *Drug Metab Rev.* (2000) 32:307–26. doi: 10.1081/DMR-100102336
19. Roy D, Wong PKY, Engelbrecht RS, Chian ESK. Mechanism of enteroviral inactivation by ozone. *Appl Environ Microbiol.* (1981) 41:718–23. doi: 10.1128/AEM.41.3.718-723.1981
20. Jiang HJ, Chen N, Shen ZQ, Yin J, Qiu ZG, Miao J, et al. Inactivation of poliovirus by ozone and the impact of ozone on the viral genome. *Biomed Environ Sci.* (2019) 32:324–33. doi: 10.3967/bes2019.044
21. Murray BK, Ohmine S, Tomer DP, Jensen KJ, Johnson FB, Kirsi JJ, et al. Virion disruption by ozone-mediated reactive oxygen species. *J Virol Methods.* (2008) 153:74–7. doi: 10.1016/j.jviromet.2008.06.004
22. Rowen RJ, Robins H. A plausible "penny" costing effective treatment for corona virus - ozone therapy. *J Infect Dis Epidemiol.* (2020) 6:113. doi: 10.23937/2474-3658/1510113
23. Hernández A, Papadakos PJ, Torres A, González DA, Vives M, Ferrando C, et al. Two known therapies could be useful as adjuvant therapy in critical patients infected by COVID-19. Dos terapias conocidas podrían ser efectivas como adyuvantes en el paciente crítico infectado por COVID-19. *Rev Esp Anestesiol Reanim.* (2020) 67:245–52. doi: 10.1016/j.redar.2020.03.004
24. Italian "Istituto Superiore di Sanità" biocide COVID-19 working group. *Raccomandazioni ad interim sulla sanificazione di strutture non sanitarie nell'attuale emergenza COVID-19: superfici, ambienti interni e abbigliamento*. Version 15th May 2020. Available online at: https://www.iss.it/rapporti-covid-19/-/asset_publisher/btw1J82wtYzH/content/id/5392909 (accessed July 06, 2020).
25. Ricevuti G, Franzini M, Valdenassi L. Oxygen-ozone immunoceutical therapy in COVID-19 outbreak: facts and figures. *Ozone Therapy.* (2020) 5:9014. doi: 10.4081/ozone.2020.9014
26. Yan T, Xiao R, Lin G. Angiotensin-converting enzyme 2 in severe acute respiratory syndrome coronavirus and SARS-CoV-2: a double-edged sword? *FASEB J.* (2020) 34:6017–26. doi: 10.1096/fj.202000782
27. Zhang H, Penninger JM, Li Y, Zhong N, Slutsky AS. Angiotensin-converting enzyme 2 (ACE2) as a SARS-CoV-2 receptor: molecular mechanisms and potential therapeutic target. *Intensive Care Med.* (2020) 46:586–90. doi: 10.1007/s00134-020-05985-9
28. Scientific Society of Oxygen Ozone Therapy website. Available online at: http://backoffice.ossigenoozono.it/Multimedia/Content/LETTERA_UFFICIALE_ISS.pdf (accessed March 31, 2020).
29. Scientific Society of Oxygen Ozone Therapy website—report 7. Available online at: http://backoffice.ossigenoozono.it/Multimedia/Content/Report_n._7.pdf (accessed July 02, 2020).
30. Scientific Society of Oxygen Ozone Therapy website—report 8. Available online at: http://backoffice.ossigenoozono.it/Multimedia/Content/Report_n.8_-_English.pdf (accessed July 02, 2020).
31. World Federation of Ozone Therapy website. Available online at: https://www.wfoot.org/last-news-ozone-and-covid19/ (accessed July 06, 2020).
32. Zheng Z, Dong M, Hu K. A preliminary evaluation on the efficacy of ozone therapy in the treatment of COVID-19. *J Med Virol.* (2020). doi: 10.1002/jmv.26040. [Epub ahead of print].

Conflict of Interest: MF is Director of Comunian Clinic, Gorle (BG) where ozone therapy is routinely practiced.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cell and Developmental Biology

HYPOTHESIS AND THEORY
published: 26 August 2020
doi: 10.3389/fcell.2020.00856

Repurposing Fragile X Drugs to Inhibit SARS-CoV-2 Viral Reproduction

Cara J. Westmark[1], Maki Kiso[2], Peter Halfmann[3], Pamela R. Westmark[1] and Yoshihiro Kawaoka[2,3,4]*

[1] Department of Neurology, University of Wisconsin–Madison, Madison, WI, United States, [2] Division of Virology, Department of Microbiology and Immunology, Institute of Medical Science, The University of Tokyo, Tokyo, Japan, [3] Department of Pathobiological Sciences, School of Veterinary Medicine, Influenza Research Institute, University of Wisconsin–Madison, Madison, WI, United States, [4] Department of Special Pathogens, International Research Center for Infectious Diseases, Institute of Medical Science, The University of Tokyo, Tokyo, Japan

The COVID-19 pandemic is a global health crisis that requires the application of interdisciplinary research to address numerous knowledge gaps including molecular strategies to prevent viral reproduction in affected individuals. In response to the Frontiers Research Topic, "*Coronavirus disease (COVID-19): Pathophysiology, Epidemiology, Clinical Management, and Public Health Response,*" this Hypothesis article proposes a novel therapeutic strategy to repurpose metabotropic glutamate 5 receptor ($mGluR_5$) inhibitors to interfere with viral hijacking of the host protein synthesis machinery. We review pertinent background on SARS-CoV-2, fragile X syndrome (FXS) and metabotropic glutamate receptor 5 ($mGluR_5$) and provide a mechanistic-based hypothesis and preliminary data to support testing $mGluR_5$ inhibitors in COVID-19 research.

Keywords: coronavirus, COVID-19, fragile X mental retardation protein, metabotropic glutamate receptor 5, protein synthesis

OPEN ACCESS

Edited by:
Tatsuo Shioda,
Osaka University, Japan

Reviewed by:
Juni Sarkar,
University of Southern California, United States
Yue Liu,
Cardiovascular Diseases Center, Xiyuan Hospital, China

****Correspondence:***
Cara J. Westmark
westmark@wisc.edu;
westmark@facstaff.wisc.edu

Specialty section:
This article was submitted to Molecular Medicine, a section of the journal Frontiers in Cell and Developmental Biology

Received: *18 May 2020*
Accepted: *10 August 2020*
Published: *26 August 2020*

Citation:
Westmark CJ, Kiso M, Halfmann P, Westmark PR and Kawaoka Y (2020) Repurposing Fragile X Drugs to Inhibit SARS-CoV-2 Viral Reproduction. Front. Cell Dev. Biol. 8:856. doi: 10.3389/fcell.2020.00856

INTRODUCTION

In December of 2019, an outbreak of respiratory disease began in Wuhan, China. The causative agent was a novel betacoronavirus of the same subgenus as severe acute respiratory syndrome (SARS) coronavirus (CoV) and was named SARS-CoV-2, a.k.a. novel CoV (nCoV-2019), which causes the disease coronavirus-19 (COVID-19) (Zhu et al., 2020). COVID-19 quickly spread worldwide with clinical manifestations ranging from mild respiratory symptoms to severe pneumonia and fatality. We submit this Hypothesis paper as an interdisciplinary research approach supporting a molecular-based therapeutic strategy to reduce virus reproduction in individuals infected with SARS-CoV-2. Specifically, we provide the conceptional framework to support testing metabotropic glutamate receptor 5 ($mGluR_5$) inhibitors to attenuate virus reproduction. Inhibitors of $mGluR_5$ have been extensively studied in the neurodevelopmental disorder fragile X syndrome (FXS) as well as other psychiatric disorders (Gravius et al., 2010; Michalon et al., 2012; Scharf et al., 2015; Berry-Kravis et al., 2017). Inhibition of $mGluR_5$ represses exaggerated protein synthesis that occurs in the absence of the RNA binding protein fragile X mental retardation protein (FMRP) (Bear et al., 2004; Osterweil et al., 2010). Work by Soto-Acosta and colleagues in 2018 demonstrates that FMRP represses Zika virus (ZIKV) infection by blocking viral RNA translation

(Soto-Acosta et al., 2018). Thus, we propose that mGluR$_5$ inhibitors may be a viable therapeutic strategy to interfere with the ability of SARS-CoV-2 to hijack the host cell translational machinery.

CONCEPTUAL FRAMEWORK

SARS-CoV-2

SARS-CoV-2 is a betacoronavirus of the same family as Middle East Respiratory Syndrome (MERS-CoV) and SARS-CoV (Zhu et al., 2020). Betacoronaviruses are enveloped, non-segmented, positive-sense, single-stranded RNA [ssRNA(+)] viruses of zoonotic origin that replicate in the host cell cytoplasm and induce fever and respiratory symptoms. Infection starts by attachment of the receptor binding domain of the spike protein to host cell receptors, which mediates endocytosis of the virus into the cell and release of the ssRNA(+) viral genome into the cytoplasm. The SARS-CoV-2 and SARS-CoV receptor binding domains of spike protein share angiotensin-converting enzyme 2 (ACE2) as the host cell receptor (Li et al., 2003; Li, 2015; Wan et al., 2020). Protein-protein docking experiments and molecular dynamics (MD) simulations indicate that SARS-CoV-2 binds to ACE2 with a higher affinity than SARS-CoV, but the interaction is more temperature sensitive (He et al., 2020). These data may explain why SARS-CoV-2 is more infectious than SARS-CoV and suggest that the infection ability of SARS-CoV-2 will decline faster. Once inside the host cell, the coronavirus ssRNA(+) viral genome is used as a template to synthesize viral proteins. The viral RNA (vRNA) appears to evade the host immune system by mimicking cellular mRNA. When a critical mass of new virions are manufactured, they bud at membranes of the endoplasmic reticulum and/or Golgi apparatus and are released by exocytosis.

Regarding post-transcriptional regulation of CoV RNAs, the ∼30 kb viral RNA includes a 5′-leader sequence, 5′-untranslated region (UTR), coding sequences for viral proteins, 3′-UTR and poly(A) tail (Fehr and Perlman, 2015). The 5′-proximal two-thirds of the RNA encodes the replicase mRNA that contains 2 open reading frames, ORF1a and ORF1b. The 3′ third of CoV RNA encodes structural and accessory proteins. First, the viral RNA is translated to generate viral proteins required for transcription. Translation of ORF1a yields polyprotein 1a (pp1a), and a −1 ribosomal frame shift translates ORB1b to yield pp1ab. Together these polyproteins are processed into 16 non-structural proteins, which drive viral RNA replication and subgenomic mRNA (sgmRNA) synthesis. Specifically, the ssRNA(+) viral genome is a template for synthesis of double stranded (dsRNA), which is transcribed, thereby providing new ssRNA(+) viral genomes as well as nested sets of subgenomic mRNAs (sgmRNA) that encode structural proteins. The sgmRNA, like the RNA genome, can function as a template for negative strand RNA synthesis (Wu and Brian, 2010).

Of relevance to our hypothesis, the virus needs to commandeer the host cell protein synthesis machinery in order to propagate. Protein synthesis is dependent on the interactions between *trans* factors (RNA binding proteins, RBP) and *cis*-regulatory RNA elements. Specifically, *cis*-regulatory elements in CoV RNA need to interact with host cell RBP to translate viral mRNA. The detailed RNA-protein interactions that mediate the post-transcriptional gene regulation of SARS-CoV-2 RNA remain to be determined; however, we can predict pivotal players based on current literature. We hypothesize that FMRP, which functions as a protein synthesis inhibitor, is a pivotal molecular player and that drugs under investigation to reduce exaggerated protein synthesis in FXS may be applicable to attenuate viral protein synthesis.

Fragile X Syndrome, FMRP and ZIKV Subgenomic RNA

Fragile X syndrome is a neurodevelopmental disorder clinically characterized by low IQ, autistic-like behaviors and seizures (Hagerman and Hagerman, 2002). FXS results from a mutation in the *FMR1* gene on the X chromosome, which is associated with transcriptional silencing of the *FMR1* promoter and loss of expression of FMRP (Verkerk et al., 1991). FMRP is a mRNA binding protein that associates with polysomes or non-translating ribonucleoprotein (RNP) particles and is involved in the transport, localization and translational repression of hundreds of mRNAs (Feng et al., 1997a,b; Weiler et al., 1997; Brown et al., 2001; Darnell et al., 2001; Laggerbauer et al., 2001; Li et al., 2001; Mazroui et al., 2002; Miyashiro et al., 2003; Bagni and Greenough, 2005).

In 2018, Soto-Acosta and colleagues published an article, "*Fragile X mental retardation protein is a Zika virus restriction factor that is antagonized by subgenomic flaviviral RNA*" (Soto-Acosta et al., 2018). Briefly, FMRP is a host factor that inhibits ZIKV translation by binding to the 3′-UTR of ZIKV subgenomic flavivirus RNAs (sfRNAs). The flavivirus life cycle is completely dependent on the cytoplasmic fate of one RNA species, namely the genomic vRNA, whose replication occurs entirely in the cytoplasm and does not generate any DNA intermediates. To create an environment favorable to infection, flaviviruses have evolved mechanisms to dampen antiviral processes, notably through the production of specific vRNA degradation products termed subgenomic flavivirus RNA (sfRNA). sfRNAs are RNAs produced by the viral replication machinery but do not contribute to synthesizing viral proteins and are non-infectious (Mazeaud et al., 2018; Berthoux, 2020). These sfRNAs bind to and inhibit the activity of host proteins that would normally block virus multiplication. FMRP is one of those proteins that binds to Zika sfRNA and inhibits the production of viral proteins. In the absence of FMRP, both the rate of infection and translation of viral protein increase per cell; i.e., knockdown of FMRP increases the infection rate ∼50–80%. Soto-Acosta and colleagues hypothesized that because FMRP is a known repressor of cellular mRNA translation, that translation of ZIKV is inhibited by FMRP early after infection thus reducing ZIKV infection, but as infection progresses, sfRNA antagonizes FMRP function leading to increased expression of FMRP target genes. Overall, the findings by Soto-Acosta et al. strongly suggest that FMRP plays a pivotal role in ZIKV infection and pathogenesis through regulation of protein synthesis.

Over two decades of studies elucidating the function of FMRP demonstrate that this RBP regulates cellular protein synthesis

through multiple mechanisms including stalling polyribosomes, associating with miRNA and mRNA ribonucleoprotein complexes, and regulating the formation of RNA granules including processing (P)-bodies and stress granules (Lai et al., 2020). FMRP interacts with at least 180 other proteins of which 30% are ribosomal assembly factors (Taha et al., 2020). Thus, lack of expression of this pivotal translation regulator in FXS, or sequestration of FMRP by viral RNA, is expected to have large effects on cellular protein synthesis. Negative allosteric modulation of mGluR$_5$ rescues elevated protein synthesis in mouse models of FXS and tuberous sclerosis (Michalon et al., 2012; Kelly et al., 2018).

Fragile X Syndrome, FMRP and the Immune System

Interestingly, FXS is associated with dysregulation of the immune system, with an over-representation of infectious diseases and an under-representation of autoimmune disorders (Yu et al., 2020). Patients with FXS exhibit a significantly altered cytokine profile compared to controls. Plasma protein levels of the cytokine IL-1α are elevated and numerous serum chemokines are reduced (Ashwood et al., 2010; Van Dijck et al., 2020). The reduced levels of pro-inflammatory chemokines may indicate that the FXS immune system has a decreased capacity to respond to infection. Of importance, activation of peripheral blood mononuclear cells (PBMC) from patients with FXS with a group 1 mGluR agonist results in increased production of pro-inflammatory cytokines compared to PBMC from control subjects (Careaga et al., 2014a). The increase in cytokine production can be blocked with an mGluR$_5$ antagonist. In addition to cytokine profiles, patients with FXS have an increased propensity to exhibit elevated serum anti-neuronal antibodies (43% of males) (Lisik et al., 2015). Non-human FXS models also exhibit dysregulation of the immune system. *Drosophila melanogaster Fmr1* mutants are defective in controlling bacterial infection by *S. pneumoniae* or *S. marcescens* compared to wild type flies (O'Connor et al., 2017). Peripheral immune system function appears normal in *Fmr1*^{KO} mice, but the mutant mice exhibit elevated hippocampal IL-1β and IL-6 mRNA compared to wild type controls at 4 h post-stimulation with lipopolysaccharide (Yuskaitis et al., 2010; Hodges et al., 2020). In contrast to full-mutation FXS, women carriers with the FXS premutation have an increased comorbidity of immune-mediated disorders and decreased cytokine production of GM-CSF and IL-12 (p40) compared to controls (Winarni et al., 2012; Careaga et al., 2014b; Jalnapurkar et al., 2015). Overall, these studies suggest that altered FMRP levels are associated with aberrant immune system function. It remains to be determined if persons with FXS are more susceptible to infection by SARS-CoV-2 and other viruses, and conversely, if the *FMR1* premutation is protective against viral infection.

SARS-CoV-2 Negative Sense RNA Contains a Canonical FMRP Binding Site

Fragile X mental retardation protein binds to hundreds of cellular target mRNAs and predominantly functions to reversibly stall ribosomal translocation of messages (Darnell et al., 2011). It is of interest to determine if FMRP is a host cell factor that binds to SARS-CoV-2 genomic RNA or sgRNA as part of a regulatory mechanism involved in SARS-CoV-2 mRNA translation. FMRP binds to target RNAs via G-quartet *cis*-regulatory elements through the consensus sequence 5′-DWGG $N_{(0-2)}$ DWGG $N_{(0-1)}$ DWGG $N_{(0-1)}$ DWGG-3′ where D = A, G or U and W = A or U (Darnell et al., 2001). Based on sequence analysis of the whole genome of the Wuhan seafood market pneumonia virus genome assembly (GenBank LR757995.1), we predict that FMRP binds to negative sense of SARS-CoV-2 RNA. Specifically, there is a canonical G-quartet sequence from nucleotides 6014-5996 (**Figure 1**). FMRP also binds to target RNA through kissing complex *cis*-elements with the consensus site 5′-GGGCKAAGGARK. . . . . KAGCGRCUGG-3′ where K = G or U and R = G or A (Darnell et al., 2005). We did not find any kissing complex sequences in the positive or negative sense of SARS-Cov-2 RNA. We predict that binding of FMRP to negative sense of SARS-CoV-2 RNA sequesters FMRP such that it cannot act as a translational brake for vRNA synthesis, similar to the role sfRNA plays in antagonizing FMRP function in ZIKV.

Molecular Modeling Predicts That FMRP Binds to SARS-CoV-2 Positive and Negative Sense RNAs

Understanding how SARS-CoV-2 interferes with RNA-related posttranscriptional processes could identify novel therapies (Maranon et al., 2020). We utilized the catRAPID algorithm[1] to predict RNA/protein interactions relevant to SARS-CoV-2 RNA (GenBank LR757995.1). This algorithm identifies potential interactions between protein and RNA molecules by combining the contributions of secondary structure, hydrogen binding and van der Waal's forces to generate an interaction profile (Bellucci et al., 2011; Agostini et al., 2013; Cirillo et al., 2013). First, we utilized catRAPID omics to compute which RBP are predicted to bind to positive and negative sense SARS-CoV-2 RNAs. Top-ranked RBP included several splicing and heterogeneous nuclear ribonucleoproteins (**Table 1**). FMRP exhibited an average interaction strength of 0.30 (range 0–0.95) with an average star value of 2.58 (range 2.35–2.75) for positive sense SARS-CoV-2 RNA based on 242 predicted interactions where 57 of those interactions had an intensity ≥0.5, which is indicative of high specificity for the interaction. FMRP exhibited an average interaction strength of 0.27 (range 0–0.99) with an average star value of 2.54 (range 2.34–2.74) for negative sense SARS-CoV-2 RNA based on 42 predicted interactions where 8 interactions had an intensity ≥0.5. The interaction strength (enrichment with respect to random interactions) was computed using a reference set of 100 random protein and 100 random RNA sequences having the same lengths as the molecules under investigation, and the star rating system is a score representing the sum of the catRAPID normalized propensity, the presence of RNA/DNA binding domains and disordered regions, and the presence of known RNA-binding motifs with the range of 0–3 (Agostini et al., 2013; Cirillo et al., 2013).

[1]http://s.tartaglialab.com/page/catrapid_group

Wuhan seafood market pneumonia virus genome assembly, GenBank LR757995.1

Genome position 5996-6014: 5′-CCAAACCAACCATATCCAA-3′

Canonical G-quartet sequence: 5′-DWGG N(0–2) DWGG N(0–1) DWGG N(0–1) DWGG-3′
Reverse complement 6014-5996: 5′-TTGG AT ATGG TTGG T TTGG-3′

FIGURE 1 | SARS-Cov-2 negative sense RNA contains a canonical G-quartet FMRP binding site. FMRP binds to target RNAs via G-quartet *cis*-regulatory elements through the consensus sequence 5′-DWGG $N_{(0-2)}$ DWGG $N_{(0-1)}$ DWGG $N_{(0-1)}$ DWGG-3′ where D = any nucleotide except C and W = A or U. The whole genome sequence of the Wuhan seafood market pneumonia virus genome assembly (GenBank LR757995.1) contains a canonical G-quartet sequence at nucleotides 6014-5996 of negative sense of SARS-CoV-2 RNA. The corresponding negative sense sequence is: 5′-TTGG-AT-ATGG-TTGG-T-TTGG-3′.

TABLE 1 | Top catRAPID hits for RBP that bind to SARS-CoV-2 RNA.

Positive Strand	
CSTF2	Cleavage stimulation factor subunit 2
ESRP2	Epithelial splicing regulatory protein 2
FUS	RNA-binding protein FUS
SRSF3	Serine/arginine-rich splicing factor 3
SRSF4	Serine/arginine-rich splicing factor 4
SRSF5	Serine/arginine-rich splicing factor 5
SRS10	Serine/arginine-rich splicing factor 10
SSB	Lupus La protein
YBX1	Y-box-binding protein 1
Negative Strand	
ESRP2	Epithelial splicing regulatory protein 2
HNRNPF	Heterogeneous nuclear ribonucleoprotein F
HNRNPH1	Heterogeneous nuclear ribonucleoprotein H
HNRNPH2	Heterogeneous nuclear ribonucleoprotein H2
QK1	Protein quaking
SFPQ	Splicing factor, proline- and glutamine-rich
SRSF3	Serine/arginine-rich splicing factor 3
SRSF5	Serine/arginine-rich splicing factor 5
TIA1	Nucleolysin TIA-1 isoform p40
TRA2B	Transformer-2 protein homolog beta

TABLE 2 | Top catRAPID hits of SARS-CoV-2 that bind to FMRP.

Fragment	Interaction Propensity
Positive Strand	
11365–12560	416.94
7783–8978	274.98
1216–2411	267.13
3007–4002	265.21
3604–4799	264.88
4798–5993	263.82
8380–9575	263.65
7186–8381	262.81
4201–5396	256.57
2410–3605	253.78
Negative Strand	
11365–12560	413.11
7186–8381	272.08
4798–5993	268.10
7783–8978	267.37
10171–11366	261.15
5992–7187	259.99
5395–6590	257.13
9574–10769	255.20
8380–9575	254.77
4201–5396	254.30

Second, we utilized catRAPID Global Score with uniform fragmentation to predict FMRP (GenBank AAH86957.1)/SARS-CoV-2 interactions. The Global Score predicts the overall interaction ability of a protein-RNA pair based on an algorithm trained on data generated by photoactivatable ribonucleoside-enhanced, high-throughput sequencing of RNA isolated by crosslinking and immunoprecipitation (PAR/HITS-CLIP) (Hafner et al., 2010). The Global Scores were 0.97 for positive sense and 0.84 for negative sense SARS-CoV-2 RNA and FMRP. The top 20 predicted interaction sites were between nucleotides 11,365–12,560 for both positive and negative sense SARS-CoV-2 RNA (interaction propensity range 279–417). Additional RNA fragments with the highest interaction propensities are listed in **Table 2**. The fragment of FMRP with the highest binding activity for both positive and negative sense SARS-CoV-2 RNA encompassed amino acids 311–362, which partially overlaps with a known KH RNA binding domain in FMRP (amino acids 283–325) (Siomi et al., 1994). Other FMRP protein regions with high predicted binding affinity for nucleotides 11,365–12,560 for both positive and negative sense SARS-CoV-2 RNA overlapped or partially overlapped with known Agenet (63–120) KH1 (221–280), KH2 (283–325), C-terminal (C1, 399–526), and C2 (504–586) domains as well as intervening FMRP protein sequences.

Third, the catRAPID signal localization algorithm predicted the top interactions for positive and negative sense SARS-CoV-2 RNA (**Figure 2**). The protein region of FMRP implicated in binding overlapped with the C1 region, which is an arginine-glycine-rich (RG-rich) region that participates in non-specific RNA binding (Adinolfi et al., 1999).

And fourth, we utilized catRAPID Global Score with weighted fragmentation to predict FMRP/SARS-CoV-2 interactions. The fragmentation weighted option generates interaction predictions using intact RBP (FMRP) and fragments of the RNA (100–200 nucleotides of positive or negative SARS-CoV-2 RNA), and is useful for the study of RNAs, which are larger than 1,000 nucleotides. FMRP and SARS-CoV-2 RNA are predicted

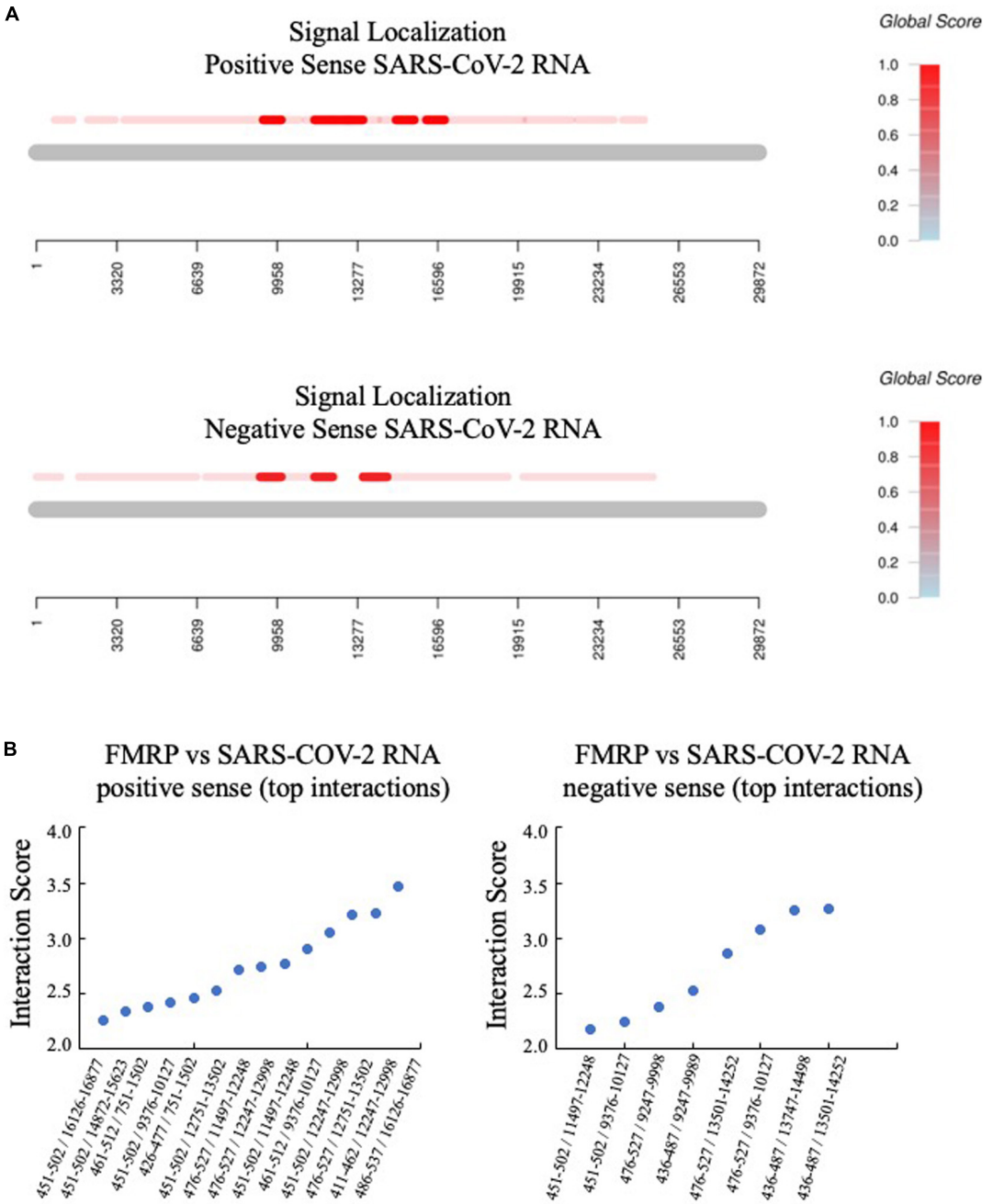


FIGURE 2 | FMRP is predicted to bind to multiple regions of SARS-CoV-2 RNA. **(A)** The catRAPID Global Score algorithm with uniform fragmentation and signal localization predicts multiple FMRP binding sites in both positive and negative sense SARS-CoV-2 RNA, with the strongest predicted binding in the region of nucleotides 9,000–17,000 (positive sense) and nucleotides 9,000–15,000 (negative sense). **(B)** The top specific interactions are graphed by protein/RNA sequence positions (*x*-axis) versus interaction score (*y*-axis). Protein and RNA coordinates are reported relative to the NCBI database.

to interact with propensities of 0.93 (positive sense) and 0.87 (negative sense). The top predicted interaction sites for positive and negative sense SARS-COV-2 are provided in the **Supplementary Figure S1**. Of note, 8 negative sense SARS-CoV-2 RNA fragments spanned the putative G-quartet region and exhibited an average interaction propensity of 5.8 ± 1.2 with FMRP. For comparison of interaction propensities, FMRP interacts with human mRNAs including *CAMK2A* (BC040457.1), *PSD-95* (U83192.1) and *APP* (BC065529.1) with global scores of 0.54, 0.68 and 0.71, respectively, using the weighted algorithm.

Overall, these molecular modeling studies indicate an overwhelming plentitude of potential interactions between FMRP and SARS-CoV-2 RNA, which remain to be experimentally validated. FMRP is predicted to bind along

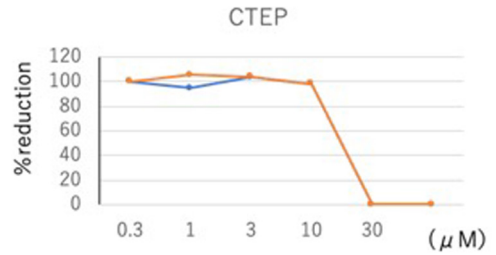


FIGURE 3 | CTEP reduces SARS-CoV-2 viral plaque load. SARS-CoV-2 virus titer was assessed in response to a 100-fold concentration range (0.3–30 μM) of the mGluR$_5$ inhibitor CTEP. Confluent VeroE6/TPMRSS2 cells seeded at a density of 5×10^5 cells per well in 6-well plates were infected with SARS-CoV-2 [approximately 80 plaque-forming units of SARS-CoV-2/UT-NCGM02/Human/2020/Tokyo (UT-NCGM02) isolated from a mild case in Tokyo]. After 30 min infection, viral inoculum was removed and cells were washed three times to remove any unbound virus before being overlaid with MEM containing a final concentration of 5% fetal bovine serum and 1.0% methyl cellulose (Sigma-Aldrich; to allow for plaque formation) along with various concentrations of CTEP. After an incubation of 2 days, the number of plaques were counted and IC_{50} values were calculated using Graphpad Prism software (Graphpad Software, La Jolla, CA, United States). CTEP reduced virus titer with an IC_{50} of 13.1 μM.

25 kB of the 29.8 kB length of positive and negative sense SARS-CoV-2 RNAs such that the RNA could act as a sink for FMRP and other RBP and prevent their normal function. The predicted interaction propensities of FMRP with SARS-CoV-2 positive and negative sense RNAs are stronger than known FMRP target mRNAs.

Repurposing mGluR$_5$ Inhibitors for Treatment of COVID-19

The leading drug target to date for FXS is the glutamate-activated, G-protein-coupled receptor mGluR$_5$, which signals through FMRP (Bear et al., 2004; Stoppel et al., 2017). The mGluRs contain a large extracellular amino terminal domain, a heptahelical transmembrane region, and an intracellular carboxy terminal domain. Negative allosteric modulators (NAMs) of mGluR$_5$ bind to the transmembrane heptahelical domain. These drugs are potent, non-competitive, selective and systematically active allosteric antagonists that are under study for a range of indications including anxiety, epilepsy, pain, depression, Parkinson's disease, gastroesophageal reflux disease, FXS, autism, and addiction (Westmark, 2014). There has been a concerted effort to repurpose mGluR$_5$ NAMs for the treatment of FXS where these drugs rescue disease phenotypes in multiple preclinical models and have been safely tested in clinical trials (Gravius et al., 2010; Michalon et al., 2012; Scharf et al., 2015; Berry-Kravis et al., 2017). Although mGluR$_5$ expression is enriched in brain tissue, the receptor is ubiquitously expressed in the body including the lungs[2]. We hypothesize that mGluR$_5$ NAMs could be a prophylactic treatment to slow viral protein synthesis in patients infected with SARS-CoV-2.

[2]The Human Protein Atlas, accessed 04/02/20 at https://www.proteinatlas.org/ENSG00000168959-GRM5.

Treatment of COVID-19 will likely require a therapeutic cocktail approach. Lead candidate drugs have been reviewed and include angiotensin receptor blockers, statins, remdesivir, chloroquine, hydroxychloroquine, lopinavir-ritonavir and interferon-beta (Kupferschmidt and Cohen, 2020). Angiotensin receptor blockers and statins upregulate ACE2, the SARS-CoV-2 host receptor, and are expected to increase the host response to infection allowing the patient to recover on their own (Fedson et al., 2020). Remdesivir shuts down viral replication by inhibiting viral RNA polymerase and has been shown to inhibit both the SARS and MERS viruses but not Ebola. Remdesivir must be given intravenously and is expensive. Chloroquine and hydroxychloroquine decrease the acidity of cellular endosomes compartments, which are involved in the degradation of foreign material. These drugs require high doses that could cause severe toxicity and many side effects. Lopinavir-ritonavir inhibits the HIV protease and has been shown effective in marmosets infected with the MERS-CoV virus. Interferon-beta regulates inflammation. A combination of lopinavir-ritonavir with interferon-beta has lessened disease severity in marmosets with MERS-CoV but could be risky for patients with severe COVID-19 and lead to more tissue damage. Other drugs under investigation for COVID-19 include corticosteroids and baricitinib, which reduce inflammation in the treatment of rheumatoid arthritis; camostat mesylate, which inhibits a human protein involved with infection; anti-viral drugs including the influenza drug favipiravir; and additional HIV antivirals (Kupferschmidt and Cohen, 2020). An alternative therapeutic strategy is to boost immunity with plasma from convalesced COVID-19 patients or monoclonal antibodies directed at SARS-CoV-2.

We propose that inclusion of mGluR$_5$ NAMs as part of a drug cocktail approach to combat COVID-19 offers the advantages of: (1) extensive preclinical research regarding its mechanism of action; (2) prior safety testing in human clinical trials of FXS; (3) numerous mGluR$_5$ NAMs available from multiple pharmaceutical countries worldwide; (4) orally dosed; (5) protein target ubiquitously expressed including the lungs; (6) less expensive to produce small molecule drugs; and (7) targets a post-transcriptional gene regulatory step in viral production not addressed by other therapies under investigation. In addition, blockade of mGluR$_5$ activity prevents an increase in proinflammatory cytokines and chemokines (Shah et al., 2012), which may quell the cytokine storm elicited by SARS-CoV-2 infection.

Preclinical Testing Strategy of mGluR$_5$ Inhibitors in SARS-COVID-2 Models

Proposed experiments to validate our hypothesis include: (1) gel mobility shift and co-immunoprecipitation assays to identify FMRP/SARS-Cov-2 RNA interactions, (2) *in vitro* translation assays to quantitate viral and cellular protein synthesis levels in the presence and absence of FMRP and mGluR$_5$ inhibitors, (3) *in vitro* assays in SARS-CoV-2 RNA-infected cells that under- and over-express FMRP to assess protein synthesis levels and virus production with/without mGluR$_5$ inhibitors, and (4)

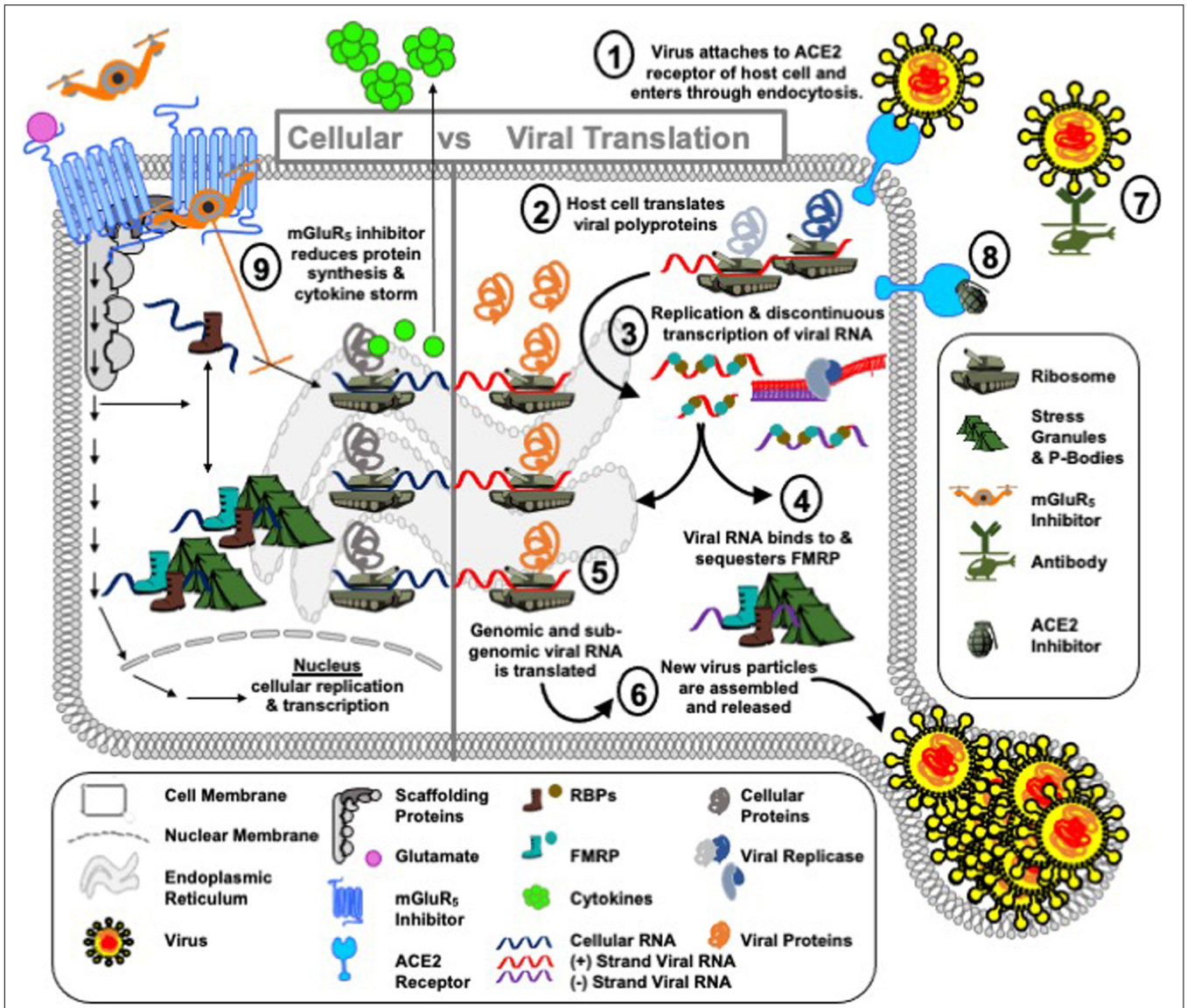


FIGURE 4 | Operation mGluR$_5$. COVID-19 is a global pandemic, i.e., a Blitzkrieg attack by SARS-CoV-2 on the human population. The port of attack for SARS-CoV-2 is the cell surface receptor angiotensin-converting enzyme 2 (ACE2) in the cells of the lungs ①. Once SARS-CoV-2 lands and breaches the cell border, the virus injects positive-sense, single-stranded RNA [ssRNA(+)] into the cell cytoplasm and immediately takes hostage of the host cell protein synthesis machinery ② to replicate and transcribe new viral RNA ③. This is accomplished by a swift and effective disarmament of the cell's shock troops, RNA binding proteins (RBPs). Shock troops is a military term for infantry formations created to lead an attack. In the RNA world, RBPs bind to RNA to either degrade, localize, store or translate messages. RBPs can bind to viral as well as cellular mRNA. In the case of viral infection, viral RNA recruits cellular RBPs to translate viral proteins at ribosomes, or sequesters cellular RBPs at other cell encampments, such as stress granules and P-bodies, to block their normal cellular function. We hypothesize that FMRP is a shock troop that is sequestered by SARS-CoV-2 RNA to prevent its normal function of acting as a brake on protein synthesis ④. In the absence of FMRP, it is predicted that the rate of viral protein synthesis ⑤ and hence further infection ⑥ are increased. Public surveillance policies and on-site diagnostics (i.e., equivalent to wartime communications and intelligence reports) to inform the public and health care professionals on viral spread are in place. Vaccines, which can mediate a rapid immune response to fight infection are in progress (i.e., cell airborne attack) ⑦. Drugs to target ACE2, the port of infection, are identified and under study (i.e., cell naval response) ⑧. What we lack in the fight against SARS-CoV-2 are drugs that support the boots on the ground, i.e., RBPs, and protect their encampments, i.e., ribosomes, stress granules and P-bodies. We propose that mGluR$_5$ inhibitors ⑨ are a potential drug therapy to combat viral hijack of the host translational infrastructure (i.e., the cell army) by slowing down protein synthesis to afford the innate immune system time to identify a viral infection and mediate an adaptive response as well as to afford the cell degradation machinery (i.e., cell marines) time to recruit and degrade viral proteins. It is anticipated that reduced protein synthesis could have negative consequences for the host cell as well as the virus; however, similar to chemotherapy that kills both healthy and cancer cells, this defensive strategy to delay advance of the stealth virus invader could buy time until the enemy can be eradicated by flanking troops. An additional potential benefit of mGluR$_5$ inhibition is reduced cytokine production, which could attenuate the COVID-19 cytokine storm.

in vivo testing of disease outcomes in a COVID-19 animal model in response to $mGluR_5$ inhibitors. An important caveat to this hypothesis is that viruses can differentially affect the host translational machinery. It will be important to test both $mGluR_5$ NAMs and positive allosteric modulators (PAMs) in preclinical studies to ascertain effects on SARS-CoV-2 RNA and protein synthesis. To our knowledge, the only study testing $mGluR_5$ drugs in a virus model was in a virus-induced temporal lobe epilepsy (TMEV) model where treatment with the PAM VU0360172 reduced acute seizures, while blocking $mGluR_5$ did not make seizure phenotypes worse (Hanak et al., 2019).

Toward validation of our model, we tested the $mGluR_5$ inhibitor 2-chloro-4-((2,5-dimethyl-1-(4-(trifluoromethoxy)phenyl)-1H-imidazol-4-yl)ethynyl)pyridine (CTEP) in an *in vitro* SARS-CoV-2 assay (**Figure 3**). CTEP is a commercially available, research-grade $mGluR_5$ inhibitor developed by Hoffmann-La Roche. This negative allosteric modulator of $mGluR_5$ acts with nanomolar affinity and greater than 1,000-fold selectivity when tested against 103 targets (Lindemann et al., 2011). CTEP has high oral bioavailability and long duration of action in animal models with a single dose lasting 18 h. CTEP reduced viral plaque load with an IC_{50} of 13.1 μM in a VeroE6/TMPRSS2 cell assay. *In vivo* testing of CTEP in a hamster COVID-19 model is in progress.

Clinical Feasibility

The old adage, "*feed a cold, starve a fever,*" may apply to treating COVID-19. Starving a fever is medical folklore for normalizing metabolism that is in overdrive. Metabolism is dependent on protein synthesis. Because virus translation dominates host cell translation at later time points of infection due to the high level of viral transcripts (Irigoyen et al., 2016), reducing protein synthesis after the onset of symptoms would be predicted to starve virus translation more than host cell translation leading to reduced virus production and affording the adaptive immune system more time to generate a response.

Inhibitors of $mGluR_5$, which have been extensively studied in both preclinical research and in clinical trials, particularly as regards FXS and Parkinson's disease (PD) (Gravius et al., 2010; Michalon et al., 2012; Scharf et al., 2015; Tison et al., 2016; Berry-Kravis et al., 2017), offer a potential repurposing strategy for COVID-19 (**Figure 4**). The FXS field has three decades of experience in mobilizing academic, pharmaceutical, biotechnology and clinical partners to repurpose drugs for a rare disorder through the efforts of FRAXA Research Foundation, the National Fragile X Foundation (NFXF) and other advocacy groups. There have been over 3,000 publications by the biomedical community to understand the role of FMRP in FXS and to test promising drugs with $mGluR_5$ as the leading drug target. This experience could be rapidly extrapolated to COVID-19. Of most importance, multiple clinical trials have been conducted in both children and adults with FXS as well as adults with PD with minimal adverse effects.

Limitations regarding repurposing $mGluR_5$ inhibitors for COVID-19 include: (1) the need for key supporting experiments regarding the mechanism, i.e., linking $mGluR_5$, FMRP and viral protein production; (2) FMRP is not the only downstream target of $mGluR_5$; (3) viral protein production is not exclusively regulated by FMRP and/or $mGluR_5$; (4) caution is required in the interpretation of the *in vitro* virus titer data in response to CTEP as weak activity is indicated by an IC_{50} of 13.1 μM; and (5) it is unknown if an effective serum concentration can be achieved in patients and if therapeutic doses will induce adverse reactions. Nonetheless, considering the dearth of therapeutic options for COVID-19 and the established safety profile of $mGluR_5$ NAMs, it is worthwhile to test clinical grade $mGluR_5$ NAMs, such as AFQ056, basimglurant and dipraglurant, in *in vitro* and *in vivo* models of COVID-19.

CONCLUSION

In conclusion, public surveillance and vaccine development for COVID-19 are on-going, but we have limited knowledge of SARS-CoV-2 post-transcriptional gene regulation and a dearth of therapeutic options. Thus, there is a critical need for the research community to rapidly mobilize to address these knowledge gaps related to COVID-19. In addition, viral infections will remain a serious threat even after COVID-19 passes. Viruses are constantly mutating and have the capacity to transmit between species. It is imperative to identify an arsenal of therapeutic options. Evidence-based research to support vaccine and drug development requires time and money to conduct rigorous and reproducible studies in preclinical models to support a hypothesis followed by extensive clinical trial validation. Thus, when currently available drugs can be repurposed for a rare disorder, or a global epidemic, it can greatly reduce the cost and time of drug validation. Targeting protein synthesis as part of a therapeutic arsenal may be a feasible broad-spectrum option to target viruses, which depend on, and cannot replicate without, the host cell translational machinery.

It remains to be determined if FMRP plays a role in protein synthesis in response to SARS-CoV-2 infection, and if $mGluR_5$ NAMs are a viable therapeutic strategy to modulate viral protein production. From a post-transcriptional gene regulation perspective, research questions that need to be addressed include: which RBP bind to and regulate synthesis of SARS-CoV-2 genomic and subgenomic RNA? Does SARS-Cov-2 RNA sequester and thereby inactivate host cell RBP such as FMRP to promote viral RNA production? Do drugs that target RBP attenuate viral replication? How do those drugs affect the immune response? Nonetheless, $mGluR_5$ NAMs have been extensively studied in non-viral models, have proven relatively safe, and may provide a rapid repurposing strategy. Similar to physical distancing and the temporary shutdown of our economy at the national level to allow public surveillance and prevent viral spread, temporary attenuation of protein synthesis at the cellular level may afford the immune system time to find and fight SARS-Cov-2.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

CW: conceptualization and original draft preparation. CW and MK: data collection. CW, PH, PW, and YK: review and editing. All authors contributed to the article and approved the submitted version.

FUNDING

CW was funded by USDA grant number 2018-67001-28266 and NIH grant number R03 HD097067. YK was funded by a grant (HHSN272201400008C) from the Center for Research on Influenza Pathogenesis, funded by the National Institute of Allergy and Infectious Diseases, and by a Research Program on Emerging and Re-emerging Infectious Disease grant (19fk0108113) from the Japan Agency for Medical Research and Development (AMED).

ACKNOWLEDGMENTS

We thank the Frontiers journals for their rapid mobilization of a COVID-19 initiative including organization of this Research Topic and the reviewers for their insightful critiques, which significantly improved the manuscript. We also thank Dr. Michael Tranfaglia (FRAXA Research Foundation), Dr. David Brown (Healx), Dr. Elizabeth Berry-Kravis (Rush University Medical Center), Dr. Fabrizio Gasparini (Novartis), Dr. Andrzej Dekundy (Merz Pharmaceuticals), and Dr. Debomoy Lahiri (Indiana University) for helpful discussions.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.00856/full#supplementary-material

REFERENCES

Adinolfi, S., Bagni, C., Musco, G., Gibson, T., Mazzarella, L., and Pastore, A. (1999). Dissecting FMR1, the protein responsible for fragile X syndrome, in its structural and functional domains. *RNA* 5, 121448–121258. doi: 10.1017/S1355838299990647

Agostini, F., Zanzoni, A., Klus, P., Marchese, D., Cirillo, D., and Tartaglia, G. G. (2013). catRAPID omics: a web server for large-scale prediction of protein-RNA interactions. *Bioinformatics* 29, 2928–2930. doi: 10.1093/bioinformatics/btt495

Ashwood, P., Nguyen, D. V., Hessl, D., Hagerman, R. J., and Tassone, F. (2010). Plasma cytokine profiles in Fragile X subjects: is there a role for cytokines in the pathogenesis? *Brain Behav. Immun.* 24, 898–902. doi: 10.1016/j.bbi.2010.01.008

Bagni, C., and Greenough, W. T. (2005). From mRNP trafficking to spine dysmorphogenesis: the roots of fragile X syndrome. *Nat. Rev. Neurosci.* 6, 376–387. doi: 10.1038/nrn1667

Bear, M. F., Huber, K. M., and Warren, S. T. (2004). The mGluR theory of fragile X mental retardation. *Trends Neurosci.* 27, 370–377. doi: 10.1016/j.tins.2004.04.009

Bellucci, M., Agostini, F., Masin, M., and Tartaglia, G. G. (2011). Predicting protein associations with long noncoding RNAs. *Nat. Methods* 8, 444–445. doi: 10.1038/nmeth.1611

Berry-Kravis, E. M., Lindemann, L., Jonch, A. E., Apostol, G., Bear, M. F., Carpenter, R. L., et al. (2017). Drug development for neurodevelopmental disorders: lessons learned from fragile X syndrome. *Nat. Rev. Drug Discov.* 17, 280–299. doi: 10.1038/nrd.2017.221

Berthoux, L. (2020). The restrictome of flaviviruses. *Virol. Sin.* doi: 10.1007/s12250-020-00208-3. [Epub ahead of print].

Brown, V., Jin, P., Ceman, S., Darnell, J. C., O'Donnell, W. T., Tenenbaum, S. A., et al. (2001). Microarray identification of FMRP-associated brain mRNAs and altered mRNA translational profiles in fragile X syndrome. *Cell* 107, 477–487. doi: 10.1016/s0092-8674(01)00568-2

Careaga, M., Noyon, T., Basuta, K., Van de Water, J., Tassone, F., Hagerman, R. J., et al. (2014a). Group I metabotropic glutamate receptor mediated dynamic immune dysfunction in children with fragile X syndrome. *J. Neuroinflammation* 11:110. doi: 10.1186/1742-2094-11-110

Careaga, M., Rose, D., Tassone, F., Berman, R. F., Hagerman, R., and Ashwood, P. (2014b). Immune dysregulation as a cause of autoinflammation in fragile X premutation carriers: link between FMRI CGG repeat number and decreased cytokine responses. *PLoS One* 9:e94475. doi: 10.1371/journal.pone.0094475

Cirillo, D., Agostini, F., Klus, P., Marchese, D., Rodriguez, S., Bolognesi, B., et al. (2013). Neurodegenerative diseases: quantitative predictions of protein-RNA interactions. *RNA* 19, 129–140. doi: 10.1261/rna.034777.112

Darnell, J. C., Fraser, C. E., Mostovetsky, O., Stefani, G., Jones, T. A., Eddy, S. R., et al. (2005). Kissing complex RNAs mediate interaction between the Fragile-X mental retardation protein KH2 domain and brain polyribosomes. *Genes Dev.* 19, 903–918. doi: 10.1101/gad.1276805

Darnell, J. C., Jensen, K. B., Jin, P., Brown, V., Warren, S. T., and Darnell, R. B. (2001). Fragile X mental retardation protein targets G quartet mRNAs important for neuronal function. *Cell* 107, 489–499. doi: 10.1016/s0092-8674(01)00566-9

Darnell, J. C., Van Driesche, S. J., Zhang, C., Hung, K. Y., Mele, A., Fraser, C. E., et al. (2011). FMRP stalls ribosomal translocation on mRNAs linked to synaptic function and autism. *Cell* 146, 247–261. doi: 10.1016/j.cell.2011.06.013

Fedson, D. S., Opal, S. M., and Rordam, O. M. (2020). Hiding in plain sight: an approach to treating patients with severe COVID-19 infection. *mBio* 11:e00398-20. doi: 10.1128/mBio.00398-20

Fehr, A. R., and Perlman, S. (2015). Coronaviruses: an overview of their replication and pathogenesis. *Methods Mol. Biol.* 1282, 1–23. doi: 10.1007/978-1-4939-2438-7_1

Feng, Y., Absher, D., Eberhart, D. E., Brown, V., Malter, H. E., and Warren, S. T. (1997a). FMRP associates with polyribosomes as an mRNP, and the I304N mutation of severe fragile X syndrome abolishes this association. *Mol. Cell* 1, 109–118. doi: 10.1016/s1097-2765(00)80012-x

Feng, Y., Gutekunst, C. A., Eberhart, D. E., Yi, H., Warren, S. T., and Hersch, S. M. (1997b). Fragile X mental retardation protein: nucleocytoplasmic shuttling and association with somatodendritic ribosomes. *J. Neurosci.* 17, 1539–1547. doi: 10.1523/jneurosci.17-05-01539.1997

Gravius, A., Pietraszek, M., Dekundy, A., and Danysz, W. (2010). Metabotropic glutamate receptors as therapeutic targets for cognitive disorders. *Curr. Top. Med. Chem.* 10, 187–206. doi: 10.2174/156802610790411018

Hafner, M., Landthaler, M., Burger, L., Khorshid, M., Hausser, J., Berninger, P., et al. (2010). Transcriptome-wide identification of RNA-binding protein and microRNA target sites by PAR-CLIP. *Cell* 141, 129–141. doi: 10.1016/j.cell.2010.03.009

Hagerman, R. J., and Hagerman, P. J. (2002). *Physical and Behavioral Phenotype*. Baltimore: John Hopkins University Press.

Hanak, T. J., Libbey, J. E., Doty, D. J., Sim, J. T., DePaula-Silva, A. B., and Fujinami, R. S. (2019). Positive modulation of mGluR5 attenuates seizures and reduces TNF-alpha(+) macrophages and microglia in the brain in a murine model of virus-induced temporal lobe epilepsy. *Exp. Neurol.* 311, 194–204. doi: 10.1016/j.expneurol.2018.10.006

He, J., Tao, H., Yan, Y., Huang, S.-Y., and Yi, X. (2020). Molecular mechanism of evolution and human infection with SARS-CoV-2. *Viruses.* 12:428. doi: 10.3390/v12040428

Hodges, S. L., Nolan, S. O., Tomac, L. A., Muhammad, I. D. A., Binder, M. S., Taube, J. H., et al. (2020). Lipopolysaccharide-induced inflammation leads to acute elevations in pro-inflammatory cytokine expression in a mouse model of Fragile X syndrome. *Physiol. Behav.* 215:112776. doi: 10.1016/j.physbeh.2019.112776

Irigoyen, N., Firth, A. E., Jones, J. D., Chung, B. Y., Siddell, S. G., and Brierley, I. (2016). High-resolution analysis of coronavirus gene expression by RNA sequencing and ribosome profiling. *PLoS Pathog.* 12:e1005473. doi: 10.1371/journal.ppat.1005473

Jalnapurkar, I., Rafika, N., Tassone, F., and Hagerman, R. (2015). Immune mediated disorders in women with a fragile X expansion and FXTAS. *Am. J. Med. Genet. A.* 167A, 190–197. doi: 10.1002/ajmg.a.36748

Kelly, E., Schaeffer, S. M., Dhamne, S. C., Lipton, J. O., Lindemann, L., Honer, M., et al. (2018). mGluR5 modulation of behavioral and epileptic phenotypes in a mouse model of tuberous sclerosis complex. *Neuropsychopharmacology* 43, 1457–1465. doi: 10.1038/npp.2017.295

Kupferschmidt, K., and Cohen, J. (2020). Race to find COVID-19 treatments accelerates. *Science* 367, 1412–1413. doi: 10.1126/science.367.6485.1412

Laggerbauer, B., Ostareck, D., Keidel, E. M., Ostareck-Lederer, A., and Fischer, U. (2001). Evidence that fragile X mental retardation protein is a negative regulator of translation. *Hum. Mol. Genet.* 10, 329–338. doi: 10.1093/hmg/10.4.329

Lai, A., Valdez-Sinon, A. N., and Bassell, G. J. (2020). Regulation of RNA granules by FMRP and implications for neurological diseases. *Traffic* 21, 454–462. doi: 10.1111/tra.12733

Li, F. (2015). Receptor recognition mechanisms of coronaviruses: a decade of structural studies. *J. Virol.* 89, 1954–1964. doi: 10.1128/jvi.02615-14

Li, W., Moore, M. J., Vasilieva, N., Sui, J., Wong, S. K., Berne, M. A., et al. (2003). Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. *Nature* 426, 450–454. doi: 10.1038/nature02145

Li, Z., Zhang, Y., Ku, L., Wilkinson, K. D., Warren, S. T., and Feng, Y. (2001). The fragile X mental retardation protein inhibits translation via interacting with mRNA. *Nucleic Acids Res.* 29, 2276–2283. doi: 10.1093/nar/29.11.2276

Lindemann, L., Jaeschke, G., Michalon, A., Vieira, E., Honer, M., Spooren, W., et al. (2011). CTEP: a novel, potent, long-acting, and orally bioavailable metabotropic glutamate receptor 5 inhibitor. *J. Pharmacol. Exp. Ther.* 339, 474–486. doi: 10.1124/jpet.111.185660

Lisik, M. Z., Gutmajster, E., and Sieron, A. L. (2015). Anti-neuronal antibodies in patients with fragile X syndrome: is there a role of autoimmunity in its pathogenesis? *Neurodegener. Dis.* 15, 45–49. doi: 10.1159/000369215

Maranon, D. G., Anderson, J., Maranon, A. G., and Wilusz, J. (2020). The interface between coronaviruses and host cell RNA biology: novel potential insights for future therapeutic intervention. *WIREs RNA*. doi: 10.1002/wrna.1614. [Epub ahead of print].

Mazeaud, C., Freppel, W., and Chatel-Chaix, L. (2018). The multiples fates of the flavivirus RNA genome during pathogenesis. *Front. Genet.* 9:595. doi: 10.3389/fgene.2018.00595

Mazroui, R., Huot, M. E., Tremblay, S., Filion, C., Labelle, Y., and Khandjian, E. W. (2002). Trapping of messenger RNA by Fragile X Mental Retardation protein into cytoplasmic granules induces translation repression. *Hum. Mol. Genet.* 11, 3007–3017. doi: 10.1093/hmg/11.24.3007

Michalon, A., Sidorov, M., Ballard, T. M., Ozmen, L., Spooren, W., Wettstein, J. G., et al. (2012). Chronic pharmacological mGlu5 inhibition corrects fragile X in adult mice. *Neuron* 74, 49–56. doi: 10.1016/j.neuron.2012.03.009

Miyashiro, K. Y., Beckel-Mitchener, A., Purk, T. P., Becker, K. G., Barret, T., Liu, L., et al. (2003). RNA cargoes associating with FMRP reveal deficits in cellular functioning in Fmr1 null mice. *Neuron* 37, 417–431. doi: 10.1016/s0896-6273(03)00034-5

O'Connor, R. M., Stone, E. F., Wayne, C. R., Marcinkevicius, E. V., Ulgherait, M., Delventhal, R., et al. (2017). A Drosophila model of Fragile X syndrome exhibits defects in phagocytosis by innate immune cells. *J. Cell Biol.* 216, 595–605. doi: 10.1083/jcb.201607093

Osterweil, E. K., Krueger, D. D., Reinhold, K., and Bear, M. F. (2010). Hypersensitivity to mGluR5 and ERK1/2 leads to excessive protein synthesis in the hippocampus of a mouse model of fragile X syndrome. *J. Neurosci.* 30, 15616–15627. doi: 10.1523/jneurosci.3888-10.2010

Scharf, S. H., Jaeschke, G., Wettstein, J. G., and Lindemann, L. (2015). Metabotropic glutamate receptor 5 as drug target for Fragile X syndrome. *Curr. Opin. Pharmacol.* 20, 124–134. doi: 10.1016/j.coph.2014.11.004

Shah, A., Silverstein, P. S., Singh, D. P., and Kumar, A. (2012). Involvement of metabotropic glutamate receptor 5, AKT/PI3K signaling and NF-kappaB pathway in methamphetamine-mediated increase in IL-6 and IL-8 expression in astrocytes. *J. Neuroinflammation* 9:52. doi: 10.1186/1742-2094-9-52

Siomi, H., Choi, M., Siomi, M. C., Nussbaum, R. L., and Dreyfuss, G. (1994). Essential role of KH domains in RNA binding: impaired RNA binding by a mutation in the KH domain of FMR1 that causes fragile X syndrome. *Cell* 77, 33–39. doi: 10.1016/0092-8674(94)90232-1

Soto-Acosta, R., Xie, X., Shan, C., Baker, C. K., Shi, P. Y., Rossi, S. L., et al. (2018). Fragile X mental retardation protein is a Zika virus restriction factor that is antagonized by subgenomic flaviviral RNA. *Elife* 7:e39023. doi: 10.7554/eLife.39023

Stoppel, L. J., Osterweil, E. K., and Bear, M. F. (2017). "Chapter 9: the mGluR theory of fragile X: from mice to men," in *Fragile X Syndrome from Genetics to Targeted Treatment*, eds R. Willemsen and F. Kooy (Amsterdam: Elsevier).

Taha, M. S., Haghighi, F., Stefansik, A., Nakhaei-Rad, S., Kazemein Jaemi, N. S., Aghyad Al Kabbani, M., et al. (2020). Novel FMRP interaction networks linked to cellular stress. *FEBS J.* doi: 10.1111/febs.15443 [Epub ahead of print].

Tison, F., Keywood, C., Wakefield, M., Durif, F., Corvol, J. C., Eggert, K., et al. (2016). A phase 2A trial of the novel mGluR5-negative allosteric modulator dipraglurant for levodopa-induced dyskinesia in Parkinson's disease. *Mov. Disord.* 31, 1373–1380. doi: 10.1002/mds.26659

Van Dijck, A., Barbosa, S., Bermudez-Martin, P., Khalfallah, O., Gilet, C., Martinuzzi, E., et al. (2020). Reduced serum levels of pro-inflammatory chemokines in fragile X syndrome. *BMC Neurol.* 20:138. doi: 10.1186/s12883-020-01715-2

Verkerk, A. J., Pieretti, M., Sutcliffe, J. S., Fu, Y. H., Kuhl, D. P., Pizzuti, A., et al. (1991). Identification of a gene (FMR-1) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome. *Cell* 65, 905–914. doi: 10.1016/0092-8674(91)90397-h

Wan, Y., Shang, J., Graham, R., Baric, R. S., and Li, F. (2020). Receptor recognition by the novel coronavirus from Wuhan: an analysis based on decade-long structural studies of SARS coronavirus. *J. Virol.* 94:e00127-20. doi: 10.1128/JVI.00127-20

Weiler, I. J., Irwin, S. A., Klintsova, A. Y., Spencer, C. M., Brazelton, A. D., Miyashiro, K., et al. (1997). Fragile X mental retardation protein is translated near synapses in response to neurotransmitter activation. *Proc. Natl. Acad. Sci. U.S.A.* 94, 5395–5400. doi: 10.1073/pnas.94.10.5395

Westmark, C. (2014). "Chapter: group 1 metabotropic glutamate receptors: a potential therapeutic target for amyloidogenic disorders," in *Metabotropic Glutamate Receptors: Molecular Mechanisms, Role in Neurological Disorders, and Pharmacological Effects*, ed. M. Foster Olive (Hauppauge, NY: Nova Science Publishers).

Winarni, T. I., Chonchaiya, W., Sumekar, T. A., Ashwood, P., Morales, G. M., Tassone, F., et al. (2012). Immune-mediated disorders among women carriers of fragile X premutation alleles. *Am. J. Med. Genet. A.* 158A, 2473–2481. doi: 10.1002/ajmg.a.35569

Wu, H. Y., and Brian, D. A. (2010). Subgenomic messenger RNA amplification in coronaviruses. *Proc. Natl. Acad. Sci. U.S.A.* 107, 12257–12262. doi: 10.1073/pnas.1000378107

Yu, K. H., Palmer, N., Fox, K., Prock, L., Mandl, K. D., Kohane, I. S., et al. (2020). The phenotypical implications of immune dysregulation in fragile X syndrome. *Eur. J. Neurol.* 27, 590–593. doi: 10.1111/ene.14146

Yuskaitis, C. J., Beurel, E., and Jope, R. S. (2010). Evidence of reactive astrocytes but not peripheral immune system activation in a mouse model of Fragile X syndrome. *Biochim. Biophys. Acta* 1802, 1006–1012. doi: 10.1016/j.bbadis.2010.06.015

Zhu, N., Zhang, D., Wang, W., Li, X., Yang, B., Song, J., et al. (2020). A novel coronavirus from patients with pneumonia in China, 2019. *N. Engl. J. Med.* 382, 727–733. doi: 10.1056/NEJMoa2001017

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 26 August 2020
doi: 10.3389/fimmu.2020.02037

The Immune Response and Immunopathology of COVID-19

*Esmaeil Mortaz[1,2], Payam Tabarsi[1], Mohammad Varahram[3], Gert Folkerts[4] and Ian M. Adcock[5,6]**

[1] Clinical Tuberculosis and Epidemiology Research Center, National Research Institute of Tuberculosis and Lung Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran, [2] Department of Immunology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran, [3] Mycobacteriology Research Center, National Research Institute of Tuberculosis and Lung Diseases (NRITLD), Masih Daneshvari Hospital, Shahid Beheshti University of Medical Sciences, Tehran, Iran, [4] Division of Pharmacology, Faculty of Science, Utrecht Institute for Pharmaceutical Sciences, Utrecht University, Utrecht, Netherlands, [5] Respiratory Section, Faculty of Medicine, National Heart and Lung Institute, Imperial College London, London, United Kingdom, [6] Priority Research Centre for Asthma and Respiratory Diseases, Hunter Medical Research Institute, The University of Newcastle, Newcastle, NSW, Australia

Coronaviruses were first discovered in the 1960s and are named due to their crown-like shape. Sometimes, but not often, a coronavirus can infect both animals and humans. An acute respiratory disease, caused by a novel coronavirus (severe acute respiratory syndrome coronavirus-2 or SARS-CoV-2 previously known as 2019-nCoV) was identified as the cause of coronavirus disease 2019 (COVID-19) as it spread throughout China and subsequently across the globe. As of 14th July 2020, a total of 13.1 million confirmed cases globally and 572,426 deaths had been reported by the World Health Organization (WHO). SARS-CoV-2 belongs to the β-coronavirus family and shares extensive genomic identity with bat coronavirus suggesting that bats are the natural host. SARS-CoV-2 uses the same receptor, angiotensin-converting enzyme 2 (ACE2), as that for SARS-CoV, the coronavirus associated with the SARS outbreak in 2003. It mainly spreads through the respiratory tract with lymphopenia and cytokine storms occuring in the blood of subjects with severe disease. This suggests the existence of immunological dysregulation as an accompanying event during severe illness caused by this virus. The early recognition of this immunological phenotype could assist prompt recognition of patients who will progress to severe disease. Here we review the data of the immune response during COVID-19 infection. The current review summarizes our understanding of how immune dysregulation and altered cytokine networks contribute to the pathophysiology of COVID-19 patients.

Keywords: coronavirus, SARS-CoV-2, SARS-CoV, IL-6, pathogenesis, cytokines storm

OPEN ACCESS

Edited by:
Alexis M. Kalergis, Pontificia Universidad Católica de Chile, Chile

Reviewed by:
Abel Viejo-Borbolla, Hannover Medical School, Germany
Jianzhong Zhu, Yangzhou University, China

***Correspondence:**
Ian M. Adcock
Ian.Adcock@imperial.ac.uk

Specialty section:
This article was submitted to Viral Immunology, a section of the journal Frontiers in Immunology

Received: *02 May 2020*
Accepted: *27 July 2020*
Published: *26 August 2020*

Citation:
Mortaz E, Tabarsi P, Varahram M, Folkerts G and Adcock IM (2020) The Immune Response and Immunopathology of COVID-19. Front. Immunol. 11:2037. doi: 10.3389/fimmu.2020.02037

INTRODUCTION

In December 2019, a novel Coronavirus (nCoV), emerged in the Huanan wet food Market, where livestock animals are also traded, in Wuhan, Hubei Province in China. However, analysis of the first 41 hospitalized patients suggests that Wuhan seafood market may not be source of novel virus spreading (1).

This resulted in an epidemic of severe pneumonia of unknown cause (2). Genomic sequencing of viral isolates from five patients with pneumonia hospitalized from December 18 to December 29, 2019, indicated the presence of a previously unknown β-CoV strain in patients (3). This nCoV has

subsequently spread from the site of the original outbreak in China and was named as SARS-CoV-2 by the World Health Organization (WHO) on January 12th 2020 and the disease as COVID-19 on 11th February 2020 (4). It was confirmed as having 75–80% resemblance to the coronavirus that caused severe acute respiratory syndrome (SARS-CoV) (5). COVID-19 currently affects 188 countries globally[1] and up to July 14th 2020 the cumulative number of confirmed cases were 13.1 million people and at least 572,426 people have died with SARS-CoV-2 infection (6). The mortality rate varies from less than 1% up to 3.7% between countries (7) compared with a mortality rate of less than 0.1% from influenza[2]. Given the origin of the first case of COVID-19, the infection was probably transmitted from animal to human.

Coronaviruses have caused three epidemics in the past two decades namely, COVID-19, SARS, and Middle East respiratory syndrome (MERS) (8). No specific antiviral therapies currently exist but efforts to develop anti-viral therapies and a vaccine are urgently needed. This review summarizes the immune response against SARS-CoV-2 and indicates areas of interest for the development of specific anti-viral therapies against SARS-CoV-2.

CORONAVIRUS

CoV belong to the genus Coronavirus in the *Coronaviridae* family. CoVs are pleomorphic RNA viruses with special crown-shape peplomers between 80 and 160 nM in size and a genome of 27–32 kb (8). Thus, enveloped CoV are some of the largest known RNA viruses (9, 10). Coronaviruses are able to infect a variety of hosts such as humans and several other vertebrates. They are associated with several respiratory and intestinal tract infections. Pulmonary coronaviruses have long been recognized as harmful pathogens in domesticated animals that also cause upper respiratory tract infections in humans (11).

Four coronavirus genera (α, β, γ, and δ) have been characterized so far, with human coronaviruses (HCoVs) detected as being in either the α (HCoV-229E and NL63) or β (MERS-CoV, SARS-CoV, HCoV-OC43, and HCoV-HKU1) genera (12). *Coronaviruses* have a high mutation rate and a high capacity to act as pathogens when present in humans and various animals presenting with a wide range of clinical features. The disease characteristics can range from an asymptomatic course to the requirement of hospitalization in an intensive care unit. *Coronaviruses* cause infections of the respiratory, gastrointestinal, hepatic, heart, renal and neurologic systems and exacerbations of lung diseases, croup and bronchiolitis (12–23).

Coronaviruses were not considered as highly pathogenic for humans until the outbreak of SARS in 2002–2003. Before these outbreaks the two most well-known types of CoV were CoV OC43 and CoV 229E that induced mild infections in immunocompromised individuals (13, 24, 25). Furthermore, 10 years after the SARS epidemic, another highly pathogenic CoV, MERS-CoV emerged in Middle Eastern countries (2).

[1]https://www.worldometers.info/coronavirus/

[2]https://www.who.int/news-room/q-a-detail/q-a-similarities-and-differences-covid-19-and-influenza

ANGIOTENSIN-CONVERTING ENZYME 2 (ACE2)

Angiotensin converting enzyme (ACE) catalyses the formation of angiotensin II from angiotensin I and, thereby, plays a key role in the control of cardio-renal function and blood pressure (26). ACE is highly expressed in the human heart, kidney, and testis consistent with its role in cardio-renal function. ACE2 is a novel gene encoding a homolog of ACE (27) that efficiently cleaves the C-terminal residue from several peptides unrelated to the renin–angiotensin system (28). Although highest ACE2 mRNA expression levels were detected in the intestinal epithelium, pulmonary ACE2 expression and function have been given extensive attention in recent years due to the findings that ACE2 serves as the receptor for SARS-CoV (29, 30) and its role in acute lung injury (31). ACE2 expression within bronchial and nasal epithelial cells is mostly localized to goblet and mucociliary cells (30). Recent evidence shows that cell entry of SARS-CoV-2 via ACE2 could be inhibited by a pharmacologic inhibitor of the cellular serine protease TMPRSS2, which is employed by SARS-CoV-2 for S protein priming (32).

Angiotensin-converting enzyme 2 acts as a binding site or receptor for the viral anchoring or spike (S) proteins present on the exterior surfaces of beta coronaviruses (33). Upon viral binding, ACE2 is released from the epithelial cell surface into the airway surface liquid (34) via cleavage by ADAM metallopeptidase domain 17 (ADAM17) and other sheddases (35, 36). ADAM17 activation also processes the membrane form of the interleukin (IL)-6 receptor (IL-6R)-α to the soluble form (sIL-6Ra) allowing gp130-mediated activation of the transcription factor STAT3 (signal transducer and activator of transcription 3) via an sIL-6Ra-IL-6 complex in a variety of IL-6R-α-negative non-immune cells including airway epithelial cells (37). STAT3 activation, in turn, induces full activation of the pro-inflammatory nuclear factor kappa B (NF-κB) pathway (37). Thus, SARS-CoV-2 infection in the respiratory tract can activate both NF-κB and STAT3 in a feedforward mechanism (IL-6 amplifier or IL-6 Amp) leading to multiple inflammatory and autoimmune diseases (37). Since IL-6 is a functional marker of cellular senescence, the age-dependent enhancement of the IL-6 Amp might correspond to the age-dependent increase in COVID-19 mortality. Furthermore, the putative driving role of IL-6 in SARS-CoV-2 induced inflammation suggests that inhibition of Janus kinases may be an attractive therapy for severe COVID-19 patients (38).

Airway surface liquid can contain catalytically active shed or soluble ACE2 (sACE2) under both stimulated and constitutive conditions (39). sACE2 acts in a feedback loop to suppress viral entry into cells and suggests that reductions in ACE2 shedding might contribute to disease pathogenesis (40).

Modulation of ACE2 expression is seen in many lung diseases including acute lung injury (ALI). ALI is induced by viral and bacterial infections and by gastro-intestinal events such as diarrhea SARS infection induces ALI following binding to airway epithelial cells, it is known that as the virus binds to ACEs, the abundance on the cell surface, mRNA expression, and the enzymatic activity of ACE2 are significantly reduced

due shedding/internalizing processes (41, 42). Interestingly in an animal model of SARS infection, binding of the virus to ACE2 results in decreased receptor expression and severe enhancement of acid aspiration pneumonia (43). Downregulation of ACE2 following SARS infection upregulates angiotensin (Ang) II which leads, in turn, to enhanced vessels permeability and induces lung injury (43). Importantly, ACE2 is endocytosed together with SARS-CoV, resulting in the reduction of ACE2 on cells, followed by an increase of serum Ang II (44). Severe lung inflammation itself may induce dysregulation of the renin-angiotensin pathway followed by ARDS development following SARS-CoV-2 infection. Indeed, SARS-CoV-induced ARDS in an animal model is prevented by inhibitors of angiotensin receptor type 1 (AT1R) (44).

Angiotensin-converting enzyme 2 is also implicated in the pathogenesis of lung fibrosis as it modulates neutrophil infiltration in the lung by inhibiting the Ang II/AT1R axis, triggering lung fibrosis (45). In addition, the expression of Ang 1–7, an ACE2-mediated anti-inflammatory metabolite of Ang II, is dysregulated in asthma suggesting a role in asthma pathogenesis (5, 46). In addition, the expression of ACE2 is down-regulated by the asthma-associated cytokine IL-13 which may account for the lower expression of ACE2 in nasal epithelial cells of asthmatic subjects (47).

Human ACE2 is the receptor for SARS- CoV (48) as well as for SARS-CoV-2 (3, 49). The binding of the SARS-CoV-2 viral S protein appears not to be as strong as that seen with the SARS virus (3). However, other studies suggest that the SARS-CoV-2 receptor binding domain (RBD) exhibits a significantly higher binding affinity for ACE2 than SARS-CoV RBD (50). Furthermore, additional reports suggest that receptors such as dipeptidyl peptidase 4 (DPP4 or CD26), which are involved in SARS and MERS infection, may also be important in SARS-CoV-2 infection (51–54).

IMMUNOPATHOLOGY OF COVID-19 DISEASE

The pathogenesis of COVID-19 is not defined but reports from many countries indicate that the virus has the same mechanism by which it enters or invades host cells as SARS-COV. The origin of SARS-CoV-2 is not well-established, however, it is established that bats are the source of related viruses and that human to human transmission plays a critical role in its pathogenesis (1, 49, 55, 56). After entering into target cells following Spike protein association with its receptor (57), viral RNA is encapsulated and polyadenylated, and encodes various structural and non-structural polypeptide genes. These polyproteins are cleaved by proteases that exhibit chymotrypsin-like activity (58, 59). Although transmembrane serine protease 2 (TMPRSS2) is the major protease associated with CoV activation and has been linked to SARS-CoV-2 activation, recent evidence from single cell RNA-sequencing (scRNA-seq) analysis shows that ACE2 and TMPRSS2 are not expressed in the same cell (30) suggesting the involvement of other proteases such as cathepsin B and L in this process.

In general, pattern recognition receptors (PRRs) recognize invading pathogens including viruses (60). Viruses elicit several key host immune responses such as increasing the release of inflammatory factors, induction and maturation of dendritic cells (DCs) and increasing the synthesis of type I interferons (IFNs), which are important in limiting viral spread (60). Both the innate and acquired immune response are activated by SARS-CoV-2. CD4 + T cells stimulate B cells to produce virus-specific antibodies including immunoglobulin (Ig)G and IgM and CD8 + T cells directly kill virus-infected cells (**Figure 1**). T helper cells produce pro-inflammatory cytokines and mediators to help the other immune cells. SARS-CoV-2 can block the host immune defense by suppressing T cell functions by inducing their programmed cell death e.g., by apoptosis. Furthermore, the host production of complement factors such as C3a and C5a and antibodies are critical in combating the viral infection (**Figure 1**) (61–64).

Viral-Track is a novel computational approach that screens scRNA-seq data for viral RNAs (65). This approach identified a major change in the bronchoalveolar lavage immune cell landscape during severe SARS-CoV-2 infection. Interestingly, Viral–Track identified co-infection of monocytes with human metapneumovirus following dampening of the IFN response.

The pathogenesis of COVID-19 is therefore as much a result of an abnormal host response or overreaction of the immune system in some patients with unknown etiology. This results in the local production of extremely high levels of a large number of inflammatory cytokines, chemokines and free radicals locally that cause severe damage to the lungs and other organs. In the worst-case scenario, systemic overspill results in multi-organ failure and even death (66, 67). Acute respiratory distress syndrome (ARDS) is the main death cause in COVID-19 (1). However, the precise reason for this being the common immunopathological event for SARS-CoV-2, SARS-CoV, and MERS-CoV infections is unclear although it probably involves the generation of a cytokine storm (68). COVID-19 infection induces pneumonia which is characterized primarily by fever, cough, dyspnea, and bilateral infiltrates on chest imaging (1, 23, 69). Edema and prominent proteinaceous exudates, vascular congestion, and inflammatory clusters with fibrinoid material and multinucleated giant cells has also been reported in lungs of COVID-19 infected patients (70).

Overall, the transcriptional footprint of SARS-CoV-2 infection is distinct from other highly pathogenic coronaviruses and common respiratory viruses such as IAV, HPIV3, and RSV. It is noteworthy that, despite a reduced IFN-I and -III response to SARS-CoV-2, recent studies show a consistent chemokine signature (71).

IMMUNE RESPONSE AGAINST CORONAVIRUS

In patients with COVID-19, the white blood cell count can vary between leukopenia, leukocytosis, and lymphopenia, although lymphopenia appears to be more common (1, 72). Importantly, the lymphocyte count is associated with increased disease severity in COVID-19 (73, 74). Lymphopenia and lower lymphocyte

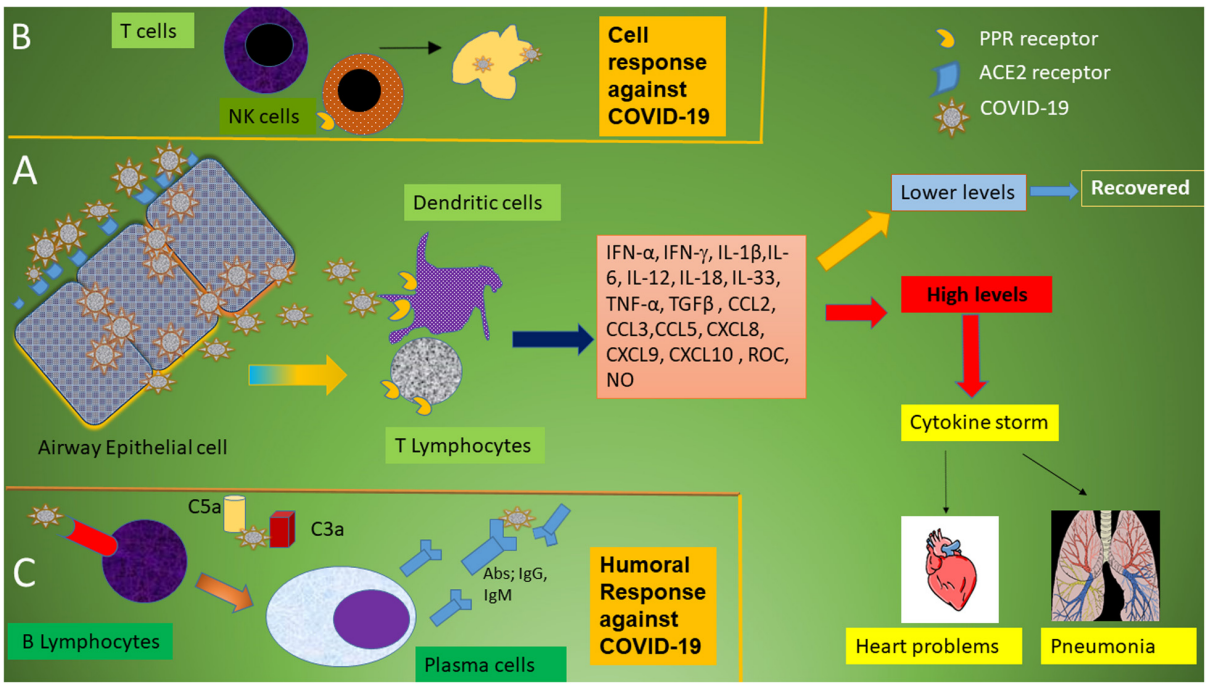


FIGURE 1 | Schematic immune responses to CoVs. **(A)** When the SARS-CoV-2 virus invades the host, it is first recognized by the angiotensin converting enzyme (ACE) 2 receptor present on respiratory epithelial cells allowing viral entry. Following viral replication within the cells, the virus is released where it is met by the host's innate immune system. T lymphocytes and dendritic cells are activated through pattern recognition receptors (PRRs) including C-type lectin-like receptors, Toll-like receptor (TLR), NOD-like receptor (NLR), and RIG-I-like receptor (RLR). The virus induces the expression of numerous inflammatory factors, maturation of dendritic cells, and the synthesis of type I interferons (IFNs) which limits the viral spread and accelerates macrophage phagocytosis of viral antigens resulting in clinical recovery. However, the N protein of SARS-CoV can help the virus escape from the immune responses and overreaction of the immune system generates high levels of inflammatory mediators and free radicals. These induce severe local damage to the lungs and other organs, and, in the worst scenario, multi-organ failure and even death. **(B)** The adaptive immune response joins the fight against the virus. T lymphocytes including CD4 + and CD8 + T cells play an important role in this defense. CD4 + T cells stimulate B cells to produce virus-specific antibodies whilst CD8 + T cells are able to directly kill virus-infected cells. T helper cells produce pro-inflammatory cytokines to help the defending cells. However, SARS-CoV-2 can inhibit T cells by inducing programmed cell death (apoptosis). **(C)** Humoral immunity including complement factors such as C3a and C5a and specific B cell-derived antibodies are also essential in combating SARS-CoV-2 infection.

counts indicated a poor prognosis in COVID-19 patients (75, 73). ICU patients suffering from COVID-19 have lymphocyte counts of 800 cells/μl and a reduced chance for survival (23). The etiology and mechanisms of lymphopenia in COVID-19 patients is unknown but SARS-like viral particles and SARS-CoV RNA has been detected in T cells suggesting a direct effect of SARS virus on T cells potentially through apoptosis (74, 75, 76).

The role of DCs in the host defense against COVID-19 unclear. During infection with SARS-CoV, antigen-presenting cell (APC) function is altered and impaired DC migration results in reduced priming of T cells. This will lead to a fewer number of virus-specific T cells within the lungs (77, 78). After initial infection with virus, lung resident respiratory DCs (rDCs) seek out the invading pathogen or antigens from infected epithelial cells, and when activated, process antigen and migrate to the draining (mediastinal and cervical) lymph nodes (DLN). Once in the DLNs, rDCs present the processed antigen in the form of MHC/peptide complex to naïve circulating T cells. Engagement of the T cell receptor (TCR) with peptide-MHC complex and additional co-stimulatory signals induce T cell activation, vigorous proliferation and migration to the site of infection (79, 80).

Cytotoxic lymphocytes (CTLs) and natural killer (NK) cells are important for the control of viral infection, and the functional exhaustion of cytotoxic lymphocytesis may increase the severity of diseases. In patients with COVID-19, the total number of NK and CTLs are decreased which is in parallel with exhaustion of their function and upregulation of NK inhibitory receptor CD94/NK group 2 member A (NKG2A) (81). After successful recovery of COVID-19 patients, the number of NK and CD8+ T cells was restored with reduced expression of NKG2A. Furthermore, there is a lower percentage of CD107a + NK, IFN-γ^+ NK, IL-2^+ NK, and TNF-α^+ NK cells in COVID-19 patients (81).

As indicated above, increased T cell apoptosis occurs in MERS infected patients (82, 83) and it is likely that this also happens in COVID-19 patients. Interestingly, the decreased number of CD4+ and CD8+ T cells in the peripheral blood of SARS-CoV-2-infected patients possess high proportions of HLA-DR (CD4 3.47%) and CD38 (CD8 39.4%) double-positive cells indicating highly activated cells (68). In addition, there was impaired activation of CD4 and CD8 cells evidenced by the appearance of CD25, CD28, and CD69 expression on these T cell subsets (84, 85). These factors may together account for the delayed

development of the adaptive immune response and prolonged virus clearance in severe human SARS-CoV infection (86).

Decreased numbers of T cells strongly correlated with the severity of the acute phase of SARS disease in humans (87, 88). Both the S and N proteins of SARS-CoV contain immunogenic epitopes that are recognized by CD4 and CD8 T cells. Viral S protein induce neutralizing antibodies and immunization with vaccines encoding the virus N-protein able to induce eosinophilic response in animals (89). In order to produce neutralizing antibodies, it is important that the viral antigen is recognized by APC as these subsequently stimulate the body's humoral immunity via virus-specific B and plasma cells (**Figure 1**). In SARS, IgM and IgG are important antibodies and the IgM antibody was detected in patient's blood 3–6 days after infection and IgG could be detected after 8 days (90, 91). The SARS-specific IgM antibodies disappeared by the end of week 12, whilst the IgG antibody can last for a long time. This suggests that generation of IgG antibodies may be essential to provide a longer term protective role (92).

Understanding the immune response to SARS-CoV-2 is crucial for vaccine development. HLA class I and II epitope pools have been used to detect CD4+ and CD8+ T cells in 100 and 70% of convalescent COVID patients (93). The CD4+ responses to the SARS-CoV-2 Spike protein correlated with the magnitude of antiviral immunoglobulin titers although T cell responses were also found against M, N, and open viral proteins. Intriguingly, 40–60% of non-SARS-CoV-2 exposed individuals also possessed CD4 + cell responses against SARS-CoV-2 indicating a degree of cross-reactivity between CoVs (93).

In addition to cell-mediated and humoral-mediated defense by the immune system, pro-inflammatory cytokine release also helps against COVID-19 infection. Effector cytokines such as IFN-γ directly inhibit viral replication and enhance antigen presentation (94). However, it has been postulated that SARS-CoV-2, due to the secretion of a novel short protein encoded by orf3b, inhibits the expression of IFNβ and enhances viral pathogenicity (95). Chemokines produced by activated T cells recruit more innate and adaptive cells to control the pathogen burden. Cytotoxic molecules such as granzyme B directly kill infected epithelial cells and help eliminate the pathogen (96–99). One of the main mechanisms for ARDS induced by SARS-CoV-2 is the cytokine storm, the deadly uncontrolled systemic inflammatory response resulting from the release of large amounts of pro-inflammatory cytokines (100).

Besides lymphocytes, other innate immune cells also play a role in the pathogenesis of COVID-19. For example, neutrophils and neutrophil-associated cytokines such as CXCL2 and CXCL8 are elevated in the blood and serum of COVID-19 patients (101). This may have prognostic value for identifying individuals at risk for developing severe disease.

The cytokine storm syndrome (CSS) is the result of an immune system running wild. In this condition the regulation of immune cells is often defective, resulting in the increased production of inflammatory proteins that can lead to organ failure and death. Among these inflammatory mediators released by immune effector cells are the cytokines IFN-α, IFN-γ, IL-1β, IL-6, IL-12, IL-18, IL-33, TNF-α, and transforming growth factor (TGF)β and chemokines such as CCL2, CCL3, CCL5, CXCL8, CXCL9, and CXCL10 (1, 66, 86, 102). Early clinical (fever, confusion) and laboratory (blood hyperferritinemia, lymphopenia, prolonged prothrombin time, elevated lactate dehydrogenase, elevated IL-6, elevated C-reactive protein, elevated soluble CD25) results from critically ill COVID-19 patients suggest the presence of a CSS causing ARDS and multi-organ failure (23, 72, 103) as seen with SARS-CoV and MERS-CoV infection (68).

Secondary hemophagocytic lymphohistiocytosis (sHLH) is an under-recognized, hyperinflammatory syndrome which is accompanied by a fulminant and fatal hyper cytokinaemia with multi-organ failure which has been reported following viral infections (104) and occurs in 3.7–4·3% of sepsis cases (105). A cytokine profile resembling sHLH is associated with COVID-19 disease severity, characterized by increased IL-2, IL-7, GCSF, IP-10, MCP-1, and MIP-α (1). All patients with severe COVID-19 should be screened for hyperinflammation such as increased ferritin, decreased platelet counts and erythrocyte sedimentation rate (106) to identify the subgroup of patients for whom immunosuppression could improve mortality. Therapeutic options include steroids, intravenous immunoglobulin, selective cytokine blockade (e.g., anakinra or tocilizumab) and JAK inhibition (107–111) and the results are eagerly awaited.

Granulocyte macrophage colony-stimulating factor (GM-CSF) is an immunoregulatory cytokine with a pivotal role in initiation and perpetuation of many inflammatory diseases. GM-CSF links T-cell-driven acute pulmonary inflammation with an autocrine, self-amplifying cytokine loop that leads to monocyte and macrophage activation. This loop has been targeted in CSS and in chronic inflammatory disorders. Importantly, the expansion of GM-CSF-expressing CD4+ T cells (Th1), CD8+ T cells, natural killer cells, and B cells are associated with disease severity in COVID-19 patients (112).

It is plausible that GM-CSF serves as an integral link between the severe pulmonary syndrome-initiating capacity of pathogenic CD4+ Th1 cells (GM-CSF+ IFNγ+) with the inflammatory signature of monocytes (CD14 + CD16 + with high expression of IL-6) (113). The potential risks associated with inhibition of GM-CSF in the context of viral infection and the challenges of doing clinical trials in this setting, highlight the fact that the mechanism(s) of induction of the cytokine storm are not well understood and that unknown genetic factors might be playing a role.

The reason for the resistance of children to COVID-19 is also unclear. However, it seems that their immune reactivity is lower than in adults and that although infants are susceptible to SARS-CoV-2 infection the severity of the disease is generally low (114). In addition, other reports have hypothesized that the lower risk of infection among children is due to differential expression of angiotensin-converting enzyme 2 (ACE2) which increases its gene expression within nasal epithelial with age (115).

A genetic predisposition to infectious viral disease has been ascribed to young and healthy adults who succumb to SARS-CoV-2 infection with resultant overt symptoms of COVID-19.

However, there is limited evidence available as yet to delineate any specific genetic markers. Dementia has been associated with an enhanced risk of COVID-19 susceptibility and higher mortality in United Kingdom patients. The apolipoprotein E (ApoE) e4 genotype is associated with an increased risk of dementia and Alzheimer's disease. Interestingly, within the United Kingdom Biobank, ApoE e4e4 homozygotes were 2.3–4.0-fold more likely to be COVID-19 test positives (OR = 2.31, 95% CI: 1.65 to 3.24) and may relate to co-expression of ApoE e4 and ACE2 within type 2 alveolar epithelial cells (116). The risks for COVID-19 mortality were not associated with chronological age or age-related comorbidities. Further studies are needed to validate these results in another cohort and to understand the mechanisms linking ApoE genotypes to COVID-19 severity.

Furthermore, there is a global effort to define the human genetics of protective immunity to SARS-CoV-2 infection (117). The goal is to compare extremes of SARS-CoV-2 susceptibility in young individuals with very severe disease and subjects with no infection despite high viral exposure.

The presence of metabolic balance syndrome/obesity, and particularly its complications, such as diabetes and hypertension, is associated with an increased propensity to develop a more serious illness, requiring hospital admission and probably invasive ventilation (111). Furthermore, patients with previous cardiovascular metabolic diseases also have a greater risk of developing severe disease highlighting the fact that the presence of comorbidities greatly affects the prognosis of the COVID-19 (118). Whether there is a genetic link to this increased risk in Caucasians is unknown but such a link is present between COVID-19 and ACE2 polymorphisms in disorders such as diabetic mellitus, cardiac diseases in Asian populations (119, 120).

In conclusion, the host immune response is the critical factor in driving COVID-19 and analysis of this response may provide a clearer picture as to how the host response impacts upon the disease severity in some individuals while most infected people only show mild symptoms or no symptoms at all. Early analysis of blood samples using scRNA-seq has revealed some interesting features (121). These include a varied IFN-stimulated response and HLA class II downregulation. Interestingly, in subjects with acute respiratory failure requiring mechanical ventilation a novel B cell-derived granulocyte population was identified. Importantly, circulating leukocytes do not express high levels of pro-inflammatory cytokines and chemokines suggesting that the COVID-19 cytokine storm is driven by cells within the lung.

Thus, the study of the host immune response from acute and convalescent individuals will provide molecular insights into mechanisms by which we may enable protection and long-term immune memory and enable the design of prophylactic and therapeutic measures to overcome future outbreaks of similar coronaviruses.

AUTHOR CONTRIBUTIONS

EM wrote the original manuscript. PT, MV, GF, and IA revised the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

IA was supported by the EPSRC (EP/T003189/1), the UK MRC (MR/T010371/1), and by the Wellcome Trust (208340/Z/17/Z).

REFERENCES

1. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet.* (2020) 15:497–506. doi: 10.1016/S0140-6736(20)30183-5
2. Anon. Seven days in medicine: 8-14 Jan 2020. *BMJ.* (2020) 368:m132. doi: 10.1136/bmj.m132
3. Lu R, Zhao X, Li J, Niu P, Yang B, Wu H, et al. Genomic characterisation and epidemiology of 2019 novel coronavirus: implications for virus origins and receptor binding. *Lancet.* (2020) 395:565–74. doi: 10.1016/S0140-6736(20)30251-8
4. European Centre for Disease Prevention and Control. *European Centre for Disease Prevention and Control data Geographical distribution of 2019-nCov cases*. (2019). Available online at: https://www.ecdc.europa.eu/en/geographical-distribution-2019-ncov-cases (accessed April 20, 2020).
5. Zheng J. SARS-CoV-2: an emerging coronavirus that causes a global threat. *Int J Biol Sci.* (2020) 16:1678–85. doi: 10.7150/ijbs.45053
6. WHO. *Update WHO Report 2020*. (2019) Available online at: https://www.who.int/emergencies/diseases/novel-coronavirus-2019/situation-reports (accessed April 20, 2020).
7. WHO. *Coronavirus Disease 2019 (COVID-19) Situation Report–52*. (2019) Geneva: World Health Organization.
8. Wit DE, Doremalen VN, Falzarano D, Munster JV. SARS and MERS: recent insights into emerging coronaviruses. *Nat Rev Microbiol.* (2016) 14:523–34. doi: 10.1038/nrmicro.2016.81
9. Siddell S, Ziebuhr J, Snijder EJ. Coronaviruses, toroviruses, and arteriviruses. Topley & Wilson's microbiology and microbial infections. *Hodder Arnold Lond.* (2005) 1:823–56.
10. Su S, Wong G, Shi W, Liu J, Lai KCA, Zhou J, et al. Epidemiology, Genetic recombination, and pathogenesis of coronaviruses. *Trends Microbiol.* (2016) 24:490–502. doi: 10.1016/j.tim.2016.03.003
11. Weiss SR, Navas-Martin S. Coronavirus pathogenesis and the emerging pathogen severe acute respiratory syndrome coronavirus. *Microbiol Mol Biol Rev.* (2005) 69:635–64. doi: 10.1128/MMBR.69.4.635-664.2005
12. Perlman S, Netland J. Coronaviruses post-SARS: update on replication and pathogenesis. *Nat Rev Microbiol.* (2009) 7:439–50. doi: 10.1038/nrmicro2147
13. Yin Y, Wunderink RR. MERS, SARS and other coronaviruses as causes of pneumonia. *Respirology.* (2018) 23:130–7. doi: 10.1111/resp.13196
14. Peiris J, Lai ST, Poon LLM, Guan Y, Yam LYC, Lim W, et al. Coronavirus as a possible cause of severe acute respiratory syndrome. *Lancet.* (2003) 361:1319–25. doi: 10.1016/S0140-6736(03)13077-2
15. Lim YX, Ng YL, Tam JP, Liu DX. Human coronaviruses: a review of virus—host interactions. *Diseases.* (2016) 4:26. doi: 10.3390/diseases40300262 doi: 10.3390/diseases4030026
16. Chan KH, Cheng VCC, Woo PCY, Lau SKP, Poon LLM, Guan Y, et al. Serological responses in patients with severe acute respiratory syndrome coronavirus infection and cross—reactivity with human coronaviruses 229E, OC43, and NL63. *Clin Diagn Lab Immunol.* (2005) 12:1317–21. doi: 10.1128/CDLI.12.11.1317-1321.2005
17. Gill EP, Dominguez EA, Greenberg SB, Atmar RL, Hogue BG, Baxter BD, et al. Development and application of an enzyme immunoassay for coronavirus OC43 antibody in acute respiratory illness. *J Clin Microbiol.* (1994) 32:2372–76. doi: 10.1128/JCM.32.10.2372-2376.1994
18. Chan CM, Tse H, Wong SSY, Woo PCY, Lau SKP, Chen L, et al. Examination of seroprevalence of coronavirus HKU1 infection with S protein—based

ELISA and neutralization assay against viral spike pseudotyped virus. *J Clin Virol.* (2009) 45:54–60. doi: 10.1016/j.jcv.2009.02.011
19. Dijkman R, Jebbink MF, El Idrissi NB, Pyrc K, Müller MA, Taco W, et al. Human coronavirus NL63and 229E seroconversion in children. *J Clin Microbiol.* (2008) 46:2368–237. doi: 10.1128/JCM.00533-08
20. Gorse GJ, Donovan MM, Patel GB, Balasubramanian S, Lusk RH. Coronavirus and other respiratory illnesses comparing older with younger adults. *Am J Med.* (2015) 128:1251.e11–e20 doi: 10.1016/j.amjmed.2015.05.034
21. Gorse GJ, O'Connor TZ, Hall SL, Vitale JN, Nichol KL. Human coronaviruses and acute respiratory illnesses in older patients with chronic obstructive pulmonary disease. *J Infect Dis.* (2009) 199:847–57. doi: 10.1086/597122
22. Walsh EE, Shin JH, Falsey AR. Clinical impact of human coronaviruses229E and OC43 infection in diverse populations. *J Infect Dis.* (2013) 208:1634–42. doi: 10.1093/infdis/jit393
23. Wang D, Hu B, Hu C, Zhu F, Liu X, Zhang J, et al. Clinical characteristics of 138 hospitalized patients with 2019 novel coronavirus-infected pneumonia in Wuhan, China. *JAMA.* (2020) 323:1061–9. doi: 10.1001/jama.2020.1585
24. Liu XD, Liang QJ, Fung ST. Human Coronavirus-229E, -OC43, -NL63, and -HKU1. *Refer Mod Life Sci.* (2020). doi: 10.1016/B978-0-12-809633-8.21501-X
25. Zaki AM, van Boheemen S, Bestebroer TM, Osterhaus AD, Fouchier RA. Isolation of a novel coronavirus from a man with pneumonia in Saudi Arabia. *N Engl J Med.* (2012) 367:1814–20. doi: 10.1056/NEJMoa1211721
26. Tikellis C, Thomas MC. Angiotensin-converting enzyme 2 (ACE2) is a key modulator of the renin angiotensin system in health and disease. *Int J Pept.* (2012) 2012:256294. doi: 10.1155/2012/256294
27. Donoghue M, Hsieh F, Baronas E, Godbout K, Gosselin M, Stagliano N, et al. A novel angiotensin-converting enzyme-related carboxypeptidase (ACE2) converts angiotensin I to angiotensin 1-9. *Circ Res.* (2000) 7:e1–9. doi: 10.1161/01.RES.87.5.e1
28. Vickers C, Hales P, Kaushik V, Dick L, Gavin G, Tang J, et al. Hydrolysis of biological peptides by human angiotensin-converting enzyme-related carboxypeptidase. *J Biol Chem.* (2002) 277:14838–43. doi: 10.1074/jbc.M200581200
29. Yang XH, Deng W, Tong Z, Liu YX, Zhang LF, Zhu H, et al. Mice transgenic for human angiotensin-converting enzyme 2 provide a model for SARS coronavirus infection. *Comp Med.* (2007) 57:450–9.
30. Sungnak W, Huang N, Bécavin C, Berg M, Queen R, Litvinukova M, et al. SARS-CoV-2 entry factors are highly expressed in nasal epithelial cells together with innate immune genes. *Nat Med.* (2020) 26:681–7. doi: 10.1038/s41591-020-0868-6
31. Imai Y, Kuba K, Rao S, Huan Y, Guo F, Guan B, et al. Angiotensin-converting enzyme 2 protects from severe acute lung failure. *Nature.* (2005) 436:112–6. doi: 10.1038/nature03712
32. Hoffmann M, Kleine-Weber H, Schroeder S, Kruger N, Herrler T, Erichse S, et al. SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor. *Cell.* (2020) 181:271–80. doi: 10.1016/j.cell.2020.02.052
33. Xu X, Chen P, Wang J, Feng J, Zhou H, Li X, et al. Evolution of the novel coronavirus from the ongoing Wuhan outbreak and modeling of its spike protein for risk of human transmission. *Life Sci.* (2020) 63:457–60. doi: 10.1007/s11427-020-1637-5
34. Waters CM, MacKinnon AC, Cummings J, Tufail-Hanif U, Jodrell D, Haslett C, et al. Increased gastrin-releasing peptide (GRP) receptor expression in tumour cells confers sensitivity to [Arg6,D-Trp7,9,NmePhe8]-substance P (6-11)-induced growth inhibition. *Br J Cancer.* (2003) 88:1808–16. doi: 10.1038/sj.bjc.6600957
35. Jia HP, Look DC, Tan P, Shi L, Hickey M, Gakhar L, et al. Ectodomain shedding of angiotensin converting enzyme 2 in human airway epithelia. *Am J Physiol Lung Cell Mol Physiol.* (2009) 297:L84–96. doi: 10.1152/ajplung.00071.2009
36. Lambert DW, Yarski M, Warner FJ, Thornhill P, Parkin ET, Smith AI, et al. Tumor necrosis factor-alpha convertase (ADAM17) mediates regulated ectodomain shedding of the severe-acute respiratory syndrome coronavirus (SARS-CoV) receptor, angiotensin-converting enzyme-2 (ACE2). *J Biol Chem.* (2005) 280:30113–9. doi: 10.1074/jbc.M505111200
37. Murakami M, Kamimura D, Hirano T. Pleiotropy and specificity: insights from the interleukin 6 family of cytokines. *Immunity.* (2019) 50:812–31. doi: 10.1016/j.immuni.2019.03.027
38. Spinelli FR, Conti F, Gadina M. HiJAKing SARS-CoV-2? The potential role of JAK inhibitors in the management of COVID-19. *Sci Immunol.* (2020) 5:eabc5367. doi: 10.1126/sciimmunol.abc5367
39. Heurich A, Hofmann-Winkler H, Gierer A, Liepold T, Jahn O, Pöhlman S. TMPRSS2 and ADAM17 Cleave ACE2 differentially and only proteolysis by TMPRSS2 augments entry driven by the severe acute respiratory syndrome coronavirus spike protein. *J Virol.* (2014) 88:1293–307. doi: 10.1128/JVI.02202-13
40. Alhenc-Gelas F, Drueke BT. Blockade of SARS-CoV-2 infection by recombinant soluble ACE2. *Kidney Int.* (2020) 97:1091–3. doi: 10.1016/j.kint.2020.04.009
41. Glowacka I, Bertram S, Herzog P, Pfefferle S, Steffen I, Muench MO, et al. Differential downregulation of ACE2 by the spike proteins of severe acute respiratory syndrome coronavirus and human coronavirus NL63. *J Virol.* (2010) 84:1198–205. doi: 10.1128/JVI.01248-09
42. Dijkman R, Jebbink MF, Deijs M, Milewska A, Pyrc K, Buelow E, et al. Replication-dependent downregulation of cellular angiotensin- converting enzyme 2 protein expression by human coronavirus NL63. *J Gen Virol.* (2012) 93:1924–9. doi: 10.1099/vir.0.043919-0
43. Kuba K, Imai Y, Rao S, Gao H, Guo F, Guan B, et al. A crucial role of angiotensin converting enzyme 2 (ACE2) in SARS coronavirus-induced lung injury. *Nat Med.* (2005) 11:875–9. doi: 10.1038/nm1267
44. Gheblawi M, Wang K, Viveiros A, Nguyen Q, Zhong J, Turner JA, et al. Angiotensin-converting enzyme 2: SARS-CoV-2 receptor and regulator of the renin-angiotensin system. Celebrating the 20th anniversary of the discovery of ACE2. *Circ Res.* (2020) 126:317015. doi: 10.1161/CIRCRESAHA.120.317015
45. Magalhaes GS, Rodrigues-Machado MG, Motta-Santos D, Silva AR, Caliari MV, Prata LO, et al. Angiotensin-(1-7) attenuates airway remodelling and hyperresponsiveness in a model of chronic allergic lung inflammation. *Br J Pharmacol.* (2015) 172:2330–42. doi: 10.1111/bph.13057
46. El-Hashim AZ, Renno WM, Raghupathy R, Abduo HT, Akhtar S, Benter IF. Angiotensin-(1-7) inhibits allergic inflammation, via the MAS1 receptor, through suppression of ERK1/2- and NF-kappaB-dependent pathways. *Br J Pharmacol.* (2012) 166:1964–76. doi: 10.1111/j.1476-5381.2012.01905.x
47. Jackson DJ, Busse WW, Bacharier LB, Kattan M, O'Connor GT, Wood RA, et al. Association of respiratory allergy, asthma and expression of the SARS-CoV-2 Receptor, ACE2. *J Allergy Clin Immunol.* (2020) 146:203–6.e3. doi: 10.1016/j.jaci.2020.04.009
48. Li W, Moore MJ, Vasilieva N, Sui J, Wong SW, Berne MA, et al. Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. *Nature.* (2003) 426:450–4. doi: 10.1038/nature02145
49. Wan Y, Shang J, Graham R, Baric RS, Li F. Receptor recognition by novel coronavirus from Wuhan: an analysis based on decade-long structural studies of SARS. *J Virol.* (2020) 94:7. doi: 10.1128/JVI.00127-20
50. Tai W, He L, Zhang X, Pu J, Voronin D, Jiang S, et al. Characterization of the receptor-binding domain (RBD) of 2019 novel coronavirus: implication for development of RBD protein as a viral attachment inhibitor and vaccine. *Cell Mol Immunol.* (2020) 17:613–20. doi: 10.1038/s41423-020-0400-4
51. Dong N, Yang X, Ye L, Chen K, Chan EW-C, Yang M, et al. Genomic and protein structure modelling analysis depicts the origin and infectivity of 2019-nCoV, a new coronavirus which caused a pneumonia outbreak in Wuhan, China. *bioRxiv.* (2020). . doi: 10.1101/2020.01.20.913368
52. Raj VS, Mou H, Smits SL, Dekkers WHD, Müller AM, Dijkman R, et al. Dipeptidyl peptidase 4 is a functional receptor for the emerging human coronavirus-EMC. *Nature.* (2013) 495:251–4. doi: 10.1038/nature12005
53. Pinheiro MM, Stoppa CL, Valduga CJ, Okuyama CE, Gorjão R, Pereira RMS, et al. Sitagliptin inhibits human lymphocyte proliferation and Th1/Th17 differentiation in vitro. *Eur J Pharm Sci.* (2017) 100:17–24. doi: 10.1016/j.ejps.2016.12.040
54. Sheikh V, Zamani A, Mahabadi-Ashtiyani E, Tarokhian H, Borzouei S, Alahgholi-Hajibehzad M. Decreased regulatory function of CD4+ CD25+ CD45RA+ T cells and impaired IL-2 signalling pathway in patients with type 2 diabetes mellitus. *Scand J Immunol.* (2018) 88:e12711. doi: 10.1111/sji.12711

55. Jia HP, Look DC, Shi L, Hickey M, Pewe L, Netland J, et al. ACE2 receptor expression and severe acute respiratory syndrome coronavirus infection depend on differentiation of human airway epithelia. *J Virol.* (2005) 79:14614–21. doi: 10.1128/JVI.79.23.14614-14621.2005
56. Zhou P, Yang XL, Wang XG, Hu B, Zhang L, Zhang W, et al. A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature.* (2020) 579:270–3.
57. Tortorici MA, Veesler D. Structural insights into coronavirus entry. *Adv Virus Res.* (2019) 105:93–116. doi: 10.1016/bs.aivir.2019.08.002
58. Coronavirinae. (2019). Available online at: https://viralzone.expasy.org/785 (accessed February 5, 2019).
59. Lambeir AM, Durinx C, Scharpe S, De Meester I. Dipeptidyl-peptidase IV from bench to bedside: an update on structural properties, functions, and clinical aspects of the enzyme DPP IV. *Crit Rev Clin Lab Sci.* (2003) 40:209–94. doi: 10.1080/713609354
60. Ben Addi A, Lefort A, Hua X, Libert F, Communi D, Ledent C, et al. Modulation of murine dendritic cell function by adenine nucleotides and adenosine: involvement of the A(2B) receptor. *Eur J Immunol.* (2008) 38:1610–20. doi: 10.1002/eji.200737781
61. Lu X, Pan J, Tao J, Guo D. SARS-CoV nucleocapsid protein antagonizes IFN-beta response by targeting initial step of IFN-beta induction pathway, and its C-terminal region is critical for the antagonism. *Virus Genes.* (2011) 42:37–45. doi: 10.1007/s11262-010-0544-x
62. Mathern DR, Heeger PS. Molecules great and small: the complement system. *Clin J Am Soc Nephrol.* (2015) 10:1636–50. doi: 10.2215/CJN.06230614
63. Traggiai E, Becker S, Subbarao K, Kolesnikova L, Uematsu Y, Gismondo MR, et al. An efficient method to make human monoclonal antibodies from memory B cells: potent neutralization of SARS coronavirus. *Nat Med.* (2004) 10:871–5. doi: 10.1038/nm1080
64. Niu P, Zhang S, Zhou P, Huang B, Deng Y, Qin K, et al. Ultrapotent human neutralizing antibody repertoires against middle east respiratory syndrome coronavirus from a recovered patient. *J Infect Dis.* (2018) 218:1249–60. doi: 10.1093/infdis/jiy311
65. Bost P, Giladi A, Liu Y, Bendjelal Y, Xu G, David EA, et al. Host-Viral infection maps reveal signatures of severe COVID-19 patients. *Cell.* (2020) 181:1475–88. doi: 10.1016/j.cell.2020.05.006
66. Channappanavar R, Perlman S. Pathogenic human coronavirus infections: causes and consequences of cytokine storm and immunopathology. *Semin Immunopathol.* (2017) 39:529–39. doi: 10.1007/s00281-017-0629-x
67. Collange O, Tacquard C, Delabranche X, Leonard-Lorant I, Ohana M, Onea M, et al. Coronavirus disease 2019: associated multiple organ damage. *Open Forum Infect Dis.* (2020) 7:ofaa249. doi: 10.1093/ofid/ofaa249
68. Xu Z, Shi L, Wang Y, Zhang J, Huang L, Zhang C, et al. Pathological findings of COVID-19 associated with acute respiratory distress syndrome. *Lancet Resp Med.* (2020) 8:420–2. doi: 10.1016/S2213-2600(20)30076-X
69. Guan WJ, Ni ZY, Hu Y, Liang WH, Ou CQ, He JX, et al. Clinical characteristics of coronavirus disease 2019 in China. *N Engl J Med.* (2020) 30:1708–20. doi: 10.1056/NEJMoa2002032
70. Tian S, Hu W, Niu L, Liu H, Xu H, Xiao YS. Pulmonary pathology of early-phase 2019 novel coronavirus (COVID-19) pneumonia in two patients with lung cancer. *J Thorac Oncol.* (2020) 15:700–4. doi: 10.1016/j.jtho.2020.02.010
71. Blanco-Melo D, Nilsson-Payant EB, Liu CW, Uhl S, Hoagland D, Møller R, et al. Imbalance host response to SARS-CoV-2 drives development of COVID-19. *Cell.* (2020) 181:1036–45. doi: 10.1016/j.cell.2020.04.026
72. Chen N, Zhou M, Dong X, Qu J, Gong F, Han Y, et al. Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study. *Lancet.* (2020) 395:507–13. doi: 10.1016/S0140-6736(20)30211-7
73. Yang X, Yu Y, Xu J, Shu H, Xia J, Liu H, et al. Clinical course and outcomes of critically ill patients with SARS-CoV-2 pneumonia in Wuhan, China: a single-centered, retrospective, observational study. *Lancet Respir Med.* (2020) 8:475–81. doi: 10.1016/S2213-2600(20)30079-5
74. Ruan Q, Yang K, Wang W, Jiang L, Song J. Clinical predictors of mortality due to COVID-19 based on an analysis of data of 150 patients from Wuhan, China. *Intensive Care Med.* (2020) 46:846–8. doi: 10.1007/s00134-020-05991-x
75. Liu J, Li S, Liu J, Liang B, Wang X, Wang H, et al. Longitudinal characteristics of lymphocyte responses and cytokine profiles in the peripheral blood of SARS-CoV-2 infected patients. *EBioMedicine.* (2020) 55:102763. doi: 10.1016/j.ebiom.2020.102763
76. Gu J, Gong E, Zhang B, Zheng J, Gao Z, Zhong Y, et al. Multiple organ infection and the pathogenesis of SARS. *J Exp Med.* (2005) 202:415–24. doi: 10.1084/jem.20050828
77. Zhao J, Zhao J, Legge K, Perlman S. Age-related increases in PGD(2) expression impair respiratory DC migration, resulting in diminished T cell responses upon respiratory virus infection in mice. *J Clin Investigat.* (2011) 121:4921–30. doi: 10.1172/JCI59777
78. Yoshikawa T, Hill T, Li K, Peters CJ, Tseng CT. Severe acute respiratory syndrome (SARS) coronavirus-induced lung epithelial cytokines exacerbate SARS pathogenesis by modulating intrinsic functions of monocyte-derived macrophages and dendritic cells. *J Virol.* (2009) 83:3039–48. doi: 10.1128/JVI.01792-08
79. Belz GT, Smith CM, Kleinert L, Reading P, Brooks A, Shortman K, et al. Distinct migrating and nonmigrating dendritic cell populations are involved in MHC class I-restricted antigen presentation after lung infection with virus. *Proc Natl Acad Sci USA.* (2004) 101:8670–5. doi: 10.1073/pnas.0402644101
80. Larsson M, Messmer D, Somersan S, Fonteneau JF, Donahoe SM, Lee M, et al. Requirement of mature dendritic cells for efficient activation of influenza A-specific memory CD8+ T cells. *J Immunol.* (2000) 165:1182–90. doi: 10.4049/jimmunol.165.3.1182
81. Norbury CC, Malide D, Gibbs JS, Bennink JR, Yewdell JW. Visualizing priming of virus-specific CD8+ T cells by infected dendritic cells in vivo. *Nat Immunol.* (2002) 3:265–71. doi: 10.1038/ni762
82. Zheng M, Gao Y, Wang G, Song G, Liu S, Sun D. Functional exhaustion of antiviral lymphocytesin COVID-19 patients. *Cell Mol Immunol.* (2020) 17:533–5. doi: 10.1038/s41423-020-0402-2
83. Chu H, Zhou J, Wong BH, Li C, Chan JF, Cheng ZS, et al. Middle east respiratory syndrome coronavirus Efficiently infects human primary T lymphocytes and activates the extrinsic and intrinsic apoptosis pathways. *J Infect Dis.* (2020) 213:904–14. doi: 10.1093/infdis/jiv380
84. Cai C, Zeng X, Ou AH, Huang Y, Zhang X. [Study on T cell subsets and their activated molecules from the convalescent SARS patients during two follow-up surveys]. *Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi Chinese J Cell Mol Immunol.* (2004) 20:322–4.
85. Yu XY, Zhang YC, Han CW, Wang P, Xue XJ, Cong YL. [Change of T lymphocyte and its activated subsets in SARS patients]. *Zhongguo Yi Xue Ke Xue Yuan Xue Bao Acta Acad Med Sin.* (2003) 25:542–6.
86. Cameron MJ, Bermejo-Martin JF, Danesh A, Muller MP, Kelvin DJ. Human immunopathogenesis of severe acute respiratory syndrome (SARS). *Virus Res.* (2008) 133:13–9. doi: 10.1016/j.virusres.2007.02.014
87. Li T, Qiu Z, Zhang L, Han Y, He W, Liu Z, et al. Significant changes of peripheral T lymphocyte subsets in patients with severe acute respiratory syndrome. *J Infect Dis.* (2004) 189:648–51. doi: 10.1086/381535
88. Li T, Qiu Z, Han Y, Wang Z, Fan H, Lu W, et al. Rapid loss of both CD4+ and CD8+ T lymphocyte subsets during the acute phase of severe acute respiratory syndrome. *Chinese Med J.* (2003) 116:985–7.
89. Bolles M, Deming D, Long K, Agnihothram S, Whitmore A, Ferris M, et al. A double-inactivated severe acute respiratory syndrome coronavirus vaccine provides incomplete protection in mice and induces increased eosinophilic proinflammatory pulmonary response upon challenge. *J Virol.* (2011) 85:12201–15. doi: 10.1128/JVI.06048-11
90. Lee HK, Lee BH, Seok SH, Baek WM, Lee YH, Kim JD, et al. Production of specific antibodies against SARS-coronavirus nucleocapsid protein without cross reactivity with human coronaviruses 229E and OC43. *J Vet Sci.* (2010) 11:165–7. doi: 10.4142/jvs.2010.11.2.165
91. Zhuoyue W, Xin Z, Xinge Y. IFA in testing specific antibody of SARS coronavirus. *South China J Prev Med.* (2003) 29:36–7.
92. Li X, Chen A, Xu A. Profile of specific antibodies to the SARS-associated coronavirus. *N Engl J Med.* (2003) 349:508–9. doi: 10.1056/NEJM200307313490520
93. Grifoni A, Weiskopf D, Ramirez SI, Mateus J, Dan JM, Rydyznski Moderbacher C, et al. Targets of T cell responses to SARS-CoV-2coronavirus in humans with COVID-19 disease and unexposed individuals. *Cell.* (2020) 181:1489–501. doi: 10.1016/j.cell.2020.05.015
94. Saha B, Jyothi Prasanna S, Chandrasekar B, Nandi D. Gene modulation and immunoregulatory roles of interferon gamma. *Cytokine.* (2010) 50:1–14. doi: 10.1016/j.cyto.2009.11.021

95. Chan JF, Kok KH, Zhu Z, Chu KK, Yuan TS, Yuen KY, et al. Genomic characterization of the 2019 novel human-pathogenic coronavirus isolated from a patient with atypical pneumonia after visiting Wuhan. *Emerg Microb Infect.* (2020) 9:221–36. doi: 10.1080/22221751.2020.1719902
96. Roman E, Miller E, Harmsen A, Wiley J, Von Andrian UH, Huston G, et al. CD4 effector T cell subsets in the response to influenza: heterogeneity, migration, and function. *J Exp Med.* (2002) 196:957–68. doi: 10.1084/jem.20021052
97. Swain SL, Agrewala JN, Brown DM, Roman E. Regulation of memory CD4 T cells: generation, localization and persistence. *Adv Exp Med Biol.* (2002) 512:113–20. doi: 10.1007/978-1-4615-0757-4_15
98. Cerwenka A, Morgan TM, Dutton RW. Naive, effector, and memory CD8 T cells in protection against pulmonary influenza virus infection: homing properties rather than initial frequencies are crucial. *J Immunol.* (1999) 163:5535–43.
99. Cerwenka A, Morgan TM, Harmsen AG, Dutton RW. Migration kinetics and final destination of type 1 and type 2 CD8 effector cells predict protection against pulmonary virus infection. *J Exp Med.* (1999) 189:423–34. doi: 10.1084/jem.189.2.423
100. Zhao M. Cytokine storm and immunomodulatory therapy in COVID-19: role of chloroquine and anti-IL-6 monoclonal antibodies. *Int J Antimicrob Agents.* (2020) 16:105982. doi: 10.1016/j.ijantimicag.2020.105982
101. Qian Z, Travanty EA, Oko L, Edeen K, Berglund A, Wang J, et al. Innate immune response of human alveolar type II cells infected with severe acute respiratory syndrome-coronavirus. *Am J Respir Cell Mol Biol.* (2013) 48:742–8. doi: 10.1165/rcmb.2012-0339OC
102. Williams AE, Chambers RC. The mercurial nature of neutrophils: still an enigma in ARDS? *Am J Physiol Lung Cell Mol Physiol.* (2014) 306:L217–30. doi: 10.1152/ajplung.00311.2013
103. Lei C, Huigo L, Wei L, Liu J, Liu K, Shang J, et al. Analysis of clinical features of 29 patients with 2019 novel coronavirus pneumonia. *Chin J Tuberc Respir Dis.* (2020) 43:E005.
104. Ramos-Casals M, Brito-Zeron P, Lopez-Guillermo A, Khamashta MA, Bosch X. Adult haemophagocytic syndrome. *Lancet.* (2014) 383:1503–16. doi: 10.1016/S0140-6736(13)61048-X
105. Karakike E, Giamarellos-Bourboulis EJ. Macrophage activation-like syndrome: a distinct entity leading to early death in sepsis. *Front Immunol.* (2019) 10:55. doi: 10.3389/fimmu.2019.00055
106. Fardet L, Galicier L, Lambotte O, Marzac C, Aumont C, Chahwan D, et al. Development and validation of the H Score, a score for the diagnosis of reactive hemophagocytic syndrome. *Arthritis Rheumatol.* (2014) 66:2613–20. doi: 10.1002/art.38690
107. Zha L, Li S, Pan L, Tefsen B, Li Y, French N, et al. Corticosteroid treatment of patients with coronavirus disease 2019 (COVID-19). *Med J Aust.* (2020) 9:416–20. doi: 10.5694/mja2.50577
108. Xie Y, Cao S, Dong H, Li Q, Chen E, Zhang W, et al. Effect of regular intravenous immunoglobulin therapy on prognosis of severe pneumonia in patients with COVID-19. *J Infect.* (2020) 81:318–56. doi: 10.1016/j.jinf.2020.03.044
109. Radbel J, Narayanan N, Bhatt PJ. Use of tocilizumab for COVID-19 infection-induced cytokine release syndrome: a cautionary case report. *Chest.* (2020) 25:24. doi: 10.1016/j.chest.2020.04.024
110. Cron RQ, Chatham WW. The question of whether to remain on therapy for chronic rheumatic diseases in the setting of the covid-19 pandemic. *J Rheumatol.* (2020) 25:jrheum.200492. doi: 10.3899/jrheum.200492
111. Simonnet A, Chetboun M, Poissy J, Raverdy V, Noulette J, Duhamel A, et al. High prevalence of obesity in severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) requiring invasive mechanical ventilation. *Obesity (Silver Spring).* (2020). doi: 10.1002/oby.22831
112. Zhou Y, Fu B, Zheng X, Wang D, Zhao C, Qi Y, et al. Pathogenic T cells and inflammatory monocytes incite inflammatory storm in severe COVID-19 patients. *Natl Sci Rev.* (2020) 13:nwaa041. doi: 10.1093/nsr/nwaa041
113. Mehta P, Porter CJ, Manson JJ, Isaacs DJ, Openshaw MJP, McInnes IB, et al. Therapeutic blockade of granulocyte macrophage colony-stimulating factor in COVID-19-associated hyperinflammation: challenges and opportunities. *Lancet.* (2020) 16:8. doi: 10.1016/S2213-2600(20)30267-8
114. Dong Y, Mo X, Hu Y, Qi X, Jiang F, Jiang Z, et al. Epidemiology of COVID-19 among children in China. *Pediatrics.* (2020) 145:e20200702. doi: 10.1542/peds.2020-0702
115. Bunyavanich S, Do A, Vicencio A. Nasal gene expression of angiotensin-converting enzyme 2 in children and adults. *JAMA.* (2020) 323:2427–9. doi: 10.1001/jama.2020.8707
116. Kuo CL, Pilling CL, Atkins LJ, Masoli HAJ, Delgado J, Kuchel AG, et al. APOE e4 genotype predicts severe COVID-19 in the UK Biobank community cohort. *J Gerontol A Biol Sci Med Sci.* (2020) 26:glaa131. doi: 10.1101/2020.05.07.20094409
117. Casanova JL, Su CHA. Global effort to define the human genetics of protective immunity to SARS-CoV-2 infection. *Cell.* (2020) 181:61194–9.
118. Li B, Yang J, Zhao F, Zhi L, Wang X, Liu L, et al. Prevalence and impact of cardiovascular metabolic diseases on COVID-19 in China. *Clin Res Cardiol.* (2020) 11:1–8.
119. Liu C, Li Y, Guan T, Lai Y, Shen Y, Zeyaweiding A, et al. ACE2 polymorphisms associated with cardiovascular risk in Uygurs with type 2 diabetes mellitus. *Cardiovas Diabetol.* (2018) 17:127. doi: 10.1186/s12933-018-0771-3
120. Fang L, Karakiulakis G, Roth M. Are patients with hypertension and diabetes mellitus at increased risk for COVID-19 infection? *Lancet.* (2020) 8:e21. doi: 10.1016/s2213-2600(20)30116-8
121. Wilk AJ, Rustagi A, Zhao NQ, Roque J, Martinez-Colon GJ, McKechnie JL, et al. A single-cell atlas of the peripheral immune response in patients with severe COVID-19. *Nat Med.* (2020) 26:1070–6. doi: 10.1038/s41591-020-0944-y

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Medicine

BRIEF RESEARCH REPORT
published: 26 August 2020
doi: 10.3389/fmed.2020.00504

Germ-Free Mice Under Two-Layer Textiles Are Fully Protected From Bacteria in Sprayed Microdroplets: A Functional *in vivo* Test Method of Facemask/Filtration Materials

Alex Rodriguez-Palacios[1,2,3,4]*, *Mathew Conger*[1,2] *and Fabio Cominelli*[1,2,3]

[1] *Division of Gastroenterology and Liver Diseases, Case Western Reserve University School of Medicine, Cleveland, OH, United States,* [2] *Germ-Free and Gut Microbiome Core, Cleveland Digestive Diseases Research Core Center, Case Western Reserve University, Cleveland, OH, United States,* [3] *Digestive Health Research Institute, University Hospitals Cleveland Medical Center, Cleveland, OH, United States,* [4] *University Hospitals Research and Education Institute, University Hospital Cleveland Medical Center, Cleveland, OH, United States*

OPEN ACCESS

Edited by:
Longxiang Su, Peking Union Medical College Hospital (CAMS), China

Reviewed by:
Rene M. Rossi, Swiss Federal Laboratories for Materials Science and Technology, Switzerland
Lieva Van Langenhove, Ghent University, Belgium

***Correspondence:**
Alex Rodriguez-Palacios
axr503@case.edu

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: *07 April 2020*
Accepted: *22 July 2020*
Published: *26 August 2020*

Citation:
Rodriguez-Palacios A, Conger M and Cominelli F (2020) Germ-Free Mice Under Two-Layer Textiles Are Fully Protected From Bacteria in Sprayed Microdroplets: A Functional in vivo Test Method of Facemask/Filtration Materials. Front. Med. 7:504. doi: 10.3389/fmed.2020.00504

Several studies have measured the effectiveness of masks at retaining particles of various sizes *in vitro*. To identify a functional *in vivo* model, herein we used germ-free (GF) mice to test the effectiveness of textiles as filtration material and droplet barriers to complement available *in vitro*-based knowledge. Herein, we report a study conducted *in vivo* with bacteria-carrying microdroplets to determine to what extent household textiles prevent contamination of GF mice in their environment. Using a recently validated spray-simulation method (mimicking a sneeze), herein we first determined that combed-cotton textiles used as two-layer-barriers covering the mouse cages prevented the contamination of all GF animals when sprayed 10–20 bacterial-droplet units/cm^2. In additional to exposure trials, the model showed that GF mice were again protected by the combed-cotton textile after the acute exposure to 10 times more droplets (20 "spray-sneezes", ∼200 bacterial-droplet units/cm^2). Overall, two-layer combed-cotton protected 100% of the GF mice from bacteria-carrying droplets ($n = 20$ exposure-events), which was significantly superior compared to 100% mouse contamination without textile coverage or when 95% partly covered ($n = 18$, Fisher-exact, $p < 0.0001$). Of relevance is that two different densities of cotton were equally effective (100%) in preventing contamination regardless of density (120–vs. 200 g/m^2; *T*-test, $p = 0.0028$), suggesting that similar density materials could prevent droplet contamination. As a practical message, we conducted a speech trial (counting numbers, 1–100) with/without the protection of the same cotton textile used as face cover. The trial illustrated that contamination of surfaces occurs at a rate of >2–6 bacteria-carrying saliva-droplets per word (2.6 droplets/cm^2, 30 cm) when speaking at 60–70 decibels and that cotton face covers fully prevent bacterial surface contamination.

Keywords: COVID-19, respiratory pandemic, cloth masks, fabrics, germ-free mouse model, public droplet safety, coronavirus in schools, decibels speech

INTRODUCTION

Since COVID-19 transmits primarily via droplet dispersion from symptomatic and asymptomatic individuals as they talk/cough/sneeze (1), the use of homemade masks is now promoted in most regions for voluntary implementation by the public (2–4). Public compliance, however, varies in part because of a spread of misinformation or disbelief regarding face masks (5). The economic impact of the COVID-19 respiratory syndrome, with doubling times between 2.4 and 5.1 days (6), will disproportionately affect poor communities (7); this is especially true given that the public has limited access to the medical personal protective equipment (including face masks) deemed effective against COVID-19 (8–20).

As an alternative to medical masks, which are in short supply due to the COVID-19 pandemic, our group (21) and others have recently quantified the benefits of textiles (8, 22). Using a spray-simulation method of bacteria-carrying macro and microdroplets, as in rapid *in-vitro* culture methods reported by our group (21) in 2020, reproducibly showed that two layers of cotton textiles were as efficient as medical mask material in reducing the environmental contamination of culture agar surfaces with sprayed droplets. In those spray-simulation studies (mimicking a sneeze), nutritious agar culture media was used to enumerate the number of sprayed microdroplets that could cross the textile.

To complement those studies, the main objective of the present study was to determine to what extent the use of germ-free (GF) mice, in a novel two-layer passive filtration GF housing system [referred to as nested isolation (23)], could be used as a functional model to characterize the benefit of textiles *in-vivo*. We hypothesized that two-layer cotton textiles used as covers could fully protect GF mice from exposure to bacteria contained in microdroplets sprayed on the other side of the textile. The goal was to quantify the potential for absolute prevention of micro-droplet dissemination into the textile-covered GF mouse cage/environment (binary data, yes/no GF mouse contamination). For the first time, GF animals are proposed as an effective *in vivo* system to assess microbial sterility, as a functional test of textiles for use as face masks and surface covers, furthering gathering data to promote a "Universal droplet reduction model" to control rapid respiratory pandemics. We also explored this further with a trial of droplet production/contamination during speech.

METHODS

Herein, we conducted studies using laboratory GF Swiss Webster mice to determine how effective household textiles are as barriers to protect the mouse environment against contamination by a mixture of bacteria-containing microdroplets using a spray simulation method (21).

Textiles

From a series of textiles recently tested in our laboratory (21), we selected 100% combed cotton (a widely available, "T-shirt material"); fabric density clustered around two types, 120 and 200 g/m^2 (GSM). This material was selected because two-layer cotton textiles were one of the most effective options at retaining sprayed liquid droplets containing bacteria during culture-based *in vitro* testing, as we demonstrated early in 2020 (21). Textiles were wrapped using surgical strategies as for surgical drape preparation, individually wrapped in ink-free paper, and autoclaved prior to use. At the time of use, the two layers were manually separated to eliminate the areas where heat had "glued" the two layers as one. Handling of materials was conducted strict aseptic measures as they are customary and previously described in our GF research facility (23).

Animals and Germ-Free Facility

The *in vivo* testing of such materials for the present study were conducted using GF Swiss Webster mice available from our Germ-Free and Gut Microbiome Core facility. The mouse line was obtained originally obtained from Taconic Biosciences Inc. (Hudson, NY). Animals were maintained using a portable static isolation strategy widely validated in our laboratory (23). Verbatim (24), as previously described in detail (23, 25), mice were maintained as GF colonies at the Animal Resource Center at Case Western Reserve University (CWRU) School of Medicine. Animals were housed in wire-topped polycarbonate shoebox cages (∼30 cm L; 15 cm W; 15 cm H) in a 12 h:12 h light:dark cycle. Autoclaved GF-grade 40–50 kGy irradiated pellet food (PMI Nutrition Int'l., LLC., Labdiet® Charles River. Vac-Pac Rodent 6/5 irradiated, 5% kcal% fat) diets and water in bottles were provided *ad libitum*. Protocols on animal handling, study designs, and housing were approved by the IACUC at CWRU in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals (26). To promote rigor and analytical reproducibility (24), GF animals were individually caged, eliminating the need to control for cyclical bias (23) or cage-clustered data (27).

Bacterial Solution

Since respiratory viruses exist in association with bacteria in respiratory fluids (28, 29), we used a bacterial-suspension spray simulation method (previously described) to quantify the number of droplets that could not be visualized but that could escape textile barriers, as recently validated by our group. In brief, we used a bacteria-carrying microdroplets spray simulation method where spray bottles were filled with an aqueous suspension of 12-probiotic-cultured dairy product (*Lactobacillus lactis, L. rhamnosus, L. plantarum, L. casei, L. acidophilus, Leuconostoc cremoris, Bifidobacterium longum, B. breve, B. lactis, Streptococcus diacetylactis*, and *Saccharomyces florentinus*, 75 ml; $3 \times 10^{6-7}$ cfu/ml, 25 ml Saliva 10^{6-7}) in 200 ml PBS (Fisher BP-399-1) to simulate a cloud of droplets produced by a sneeze (21). Probiotics are BSL-1/ "Generally Recognized As Safe" by the FDA and all experiments were conducted in BSL-2 HEPA-filtered microbiology laboratories. No human subjects were used for experimentation. The parallel lanes plating method was used to enumerate the bacterial counts in final solution (30).

Spray Simulation

Before testing, spray bottle nozzles were adjusted to produce cloud and jet-propelled droplets that match the visual architecture of droplet formation described by Bourouiba et al. (28). Specifically, we used a high-volume trigger single-v-orifice nozzle sprayer (1.0 ml per stroke) with 28/400 neck and 9-1/4-inch dip tube fitted with a filter screen (model PA-HDTS-EA, Mfr. Model # 922HL, Delta Industries, Inc.). Before conducting the experiment with animals, infrared imaging technology was used to illustrate that the spray model was composed of various liquid phases occurring within a single spray (1 ml/stroke), revealing a wide arrange of droplet sizes (right skewed distribution ranges between 20 and 900 micrometers with a peak at 70–100 micrometer) (31). In context, the size of droplets in the human sneeze ranges between 40 and 900 micrometers, with most droplets (70–100%) normally or bimodally distributed around 360–390 micrometers (32). The spray bottle ejects fluid with pressures that can reach 10 psi—sufficient to create a short burst of fluid/jet and fan cloud. In perspective, the pressure during a sneeze is between 1 psi (51.7- mmHg) in the trachea, and 2.6 psi in mouth/pharynx (135 mmHg), which can be reached in 0.1 s (33), while exhalation during strenuous activity reaches tracheal pressures of 0.03 psi (1.55 mmHg).

Droplet Quantification

To quantify the droplet exposure per surface area we used 10-mm-Petri dishes containing tryptic soy agar (56.75 cm^2 surface area/dish) with 5% defibrinated sheep blood placed on the center of cages. Cages and the agar remained covered or open for 10 min following spray bottle droplet dispersion to allow droplet landing. Before conducting the experiment with animals, infrared imaging technology was used to visually illustrate that the spray model using the methods described earlier by our group, and a liquid suspension at 46°C, on a background set at 21°C (23, 34).

Gf Housing System

Animal experiments were conducted with a system of germ-free-grade nested isolation (23) where a cage of a smaller size is nested into another one of larger capacity, each containing their respective Remay passive filtration filter as a lid for a total of two layers of filtration. In this study, the two layers of Remay filters (the cage lids) were replaced by two layers of 100% cotton material. Upon replacement of the lid's material, sets of cages (500 cm^2 floor area/cage) with individually caged (litter mate) GF mice were sprayed with the bacterial suspension, covered, or uncovered with the textiles at various distances and spray doses, 10 cm above the lid cage plane. For clarity, the "no-textile barrier controls" were the cages that remained open without a lid. Thirty seconds following the spray of the droplet-cloud, textiles were removed, and the two Remay filter lids were placed back on the nested cages.

Repeated Droplet Exposure of Mice

In short, the droplet exposure experiment was conducted in three phases (see flow chart of study overview/design in **Figure 1A**). In the **first phase**, 18 GF Swiss Webster mice (males:females, 1:1) aged 9 weeks were individually caged in our GF-grade NestIso caging system. Mice were assigned to two groups, 12 "textile-cover" and six "no-cover." Lids were temporarily removed from all cages for the spray simulation test. Twelve mouse cages were covered with the two-layer textile ("textile cover" group) while the remaining cages remained uncovered ("no cover;" no lid and no textile barrier). Each cage was then sprayed twice (spray nozzle was located at 60 cm from the cage). To determine if the droplet cloud had crossed the textile barrier, contaminating the GF environment and causing the colonization of animals, fecal samples of all animals were collected aseptically from each animal, 36 and 120 h after droplet exposure. Upon confirmation of the GF status at 120 h (5 days, end of phase one) all mice that remained GF at 120 h were then used for the **second phase** of the experiment: repeated exposure a cloud of sprayed microdroplets. Using the same strategy (covered vs. uncovered paired side-by-side cages), two thirds of the GF mice were exposed to 20 sprays (instead oftwo2; 10 times more droplets) at 60 cm, while the remaining third were left uncovered and sprayed only once at 180 cm (relevant to uncovered individuals at the recommended social distance). Feces were again measured at 36 and 120 h. Upon conformation of mouse GF status after 120 h (end of phase two), we then conducted the **third phase** experiment. Using all the mice that remained GF from phase two, phase three was conducting by covering only 95% of the cage with the two-layer textile ("partly covered" group, cages were covered, except for a corner of 5% of cage area). In this experiment, all cages were sprayed once at 90 cm. Culture of feces for confirmation of GF status was verified 120 h later.

Droplet Production During Human Speech

To put the spray experiments with GF mice into practical perspective for humans, we demonstrated the effectiveness of the cotton textile in retaining/reducing the risk of environmental contamination by oral/saliva droplets produced by one of the investigators (a healthy volunteer) during a speech trial (counting from 1 to 100 in English) conducted at 30 cm over a sterile TSA (Becton Dickinson) agar plate. Speech intensity and background noise in decibels were measured with The National Institute for Occupational Safety and Health (NIOSH) Sound Level Meter (SLM) phone app, which was placed at 90 cm (arms' reach) from the mouth. The app was developed to help individuals monitor their noise environment and promote better hearing health with accuracy of ±2 decibels. The app is freely available at app stores and from the Centers for Diseases Control and Prevention website https://www.cdc.gov/niosh/topics/noise/app.html. The speech trial, conducted by the lead investigator (healthy individual), is not considered human experimentation or subject research.

Statistical Analysis

Each time a GF mouse was exposed to a spray simulation, the event was deemed independent and referred quantitatively for binary data count statistics to as a "GF mouse exposure event." Colonization data was compared between fully covered and non-

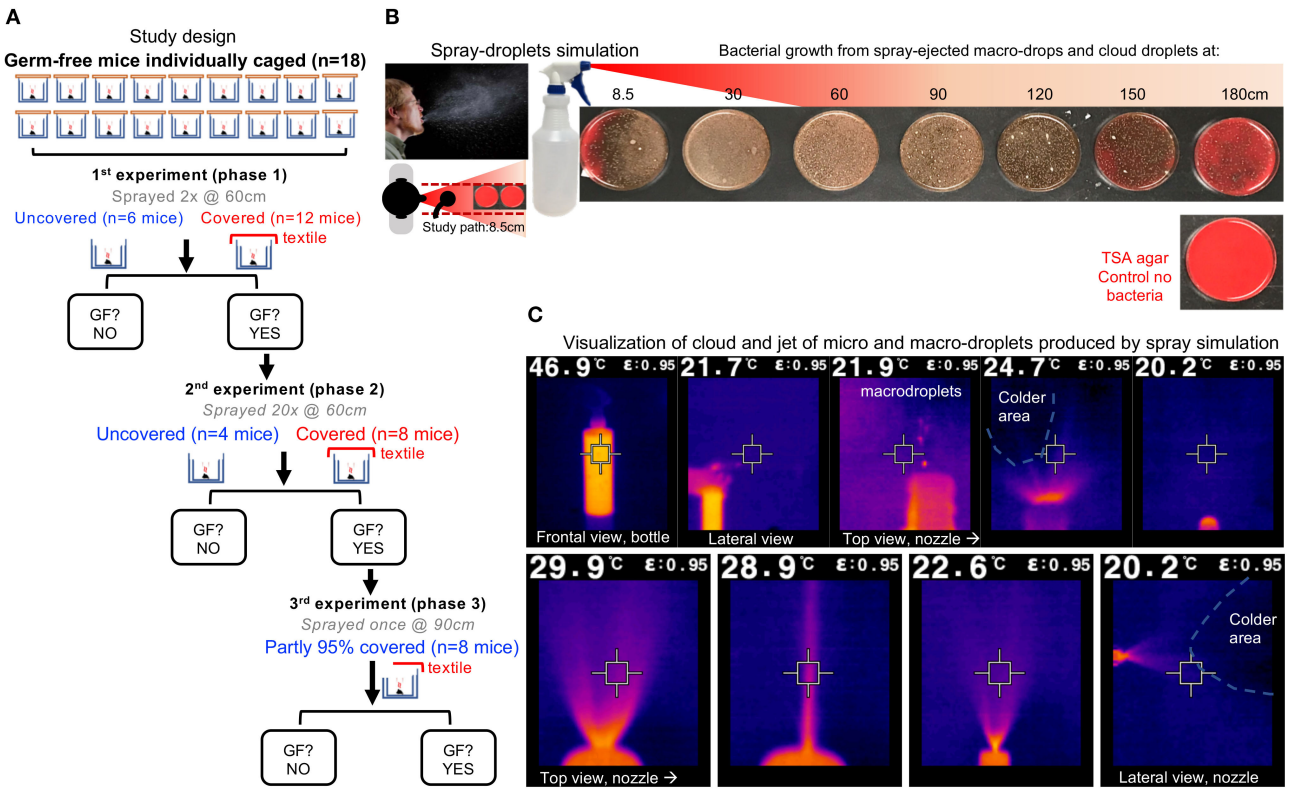


FIGURE 1 | Study overview and infrared imaging of ejection features of spray model. **(A)** Study overview. The experiment phases were conducted at different distances and with different droplet exposure doses. One mouse/cage. Each spray trail was counted as a droplet exposure event. Outcome, GF contamination 5 d after spray: yes/no. "GF?," question on whether mice remained GF, when tested after 120 h post-spray exposure to droplets, to select GF mice and continue with re-exposure experiments. If "no," end of study for those mice. If "yes," animals were GF and re-exposed to droplets. **(B)** Spray-droplet simulation model using bacterial solution as recently validated by our group for the assessment of textiles; unmodified from Rodriguez-Palacios et al. (21); open access Creative Commons Attribution (CC BY) (http://creativecommons.org/licenses/by/4.0/). Note the TSA agar plate shown has no bacteria (control no bacteria), and notice the red color of the agar changing to brown as increasing number of whitish dots (bacterial carrying microdroplets) land on the agar surface. **(C)** Infrared features of cloud-droplet ejection. Infrared imaging is based on infrared light which is electromagnetic radiation with long wavelengths that are invisible to the naked eye. Being a form of radiation, heated objects (the solution in the spray bottle) emit infrared light which contrast the lower background temperature in the room (blue or black). Electromagnetic waves carrying radiant heat energy from objects that loose heat/energy is detected by the camera, which is shown. For imaging the spray bottle was filled with liquid solution at 46.9°C (vs. Room temp of 20.2°C, humidity 70%); see high-volume trigger sprayer details in methods. The square in the center of the picture matches the color inside the square, and the temperature is shown at the top of the image. Close-up pictures illustrate the fluid ejection in proximity to the spray bottle nozzle. Notice that the solution rapidly cools down upon ejection as spray (shown as black colder area effects). Note that the simulation model resembles the features of sneeze fluid dynamics (28), with wide dispersion of high-velocity microdroplets, large heavy macro-droplets, a long-range projectile-like jet, and a large conical cloud.

or partly-covered cages using Fisher's exact test and STATA. *Post-hoc* study power statistics were computed for each analysis as recently described by our group (24). To promote open access and review, this manuscript was made available as a preprint for community contribution upon submission for peer-review (34). Sample size estimations using the open-access software G*power (24), for expected 0.1 vs. 99.9% colonization:protection, for two samples at a n1:n2 ratio of 1:1, and one-tailed $P < 0.05$, revealed that five mice per group was sufficient to achieve a power of 0.99. Since the main outcome was the presence or absence of sterility (or the permanence of GF status), the binary status (yes/no) data were analyzed using Fisher's exact test (n exposed/n contaminated by droplets) to determine if the shirt material density was a factor determining the risk of droplet retention failure (STATA, v15.1). Confidence intervals (95%) provided convey information relevant to sample size. Textile density GSM (grams /squared meter) was tested using unpaired *T*-test with Welch correction for unequal variances. Paired *T*-tests were used for textile imaging and ImageJ data analysis.

RESULTS

Infrared imaging technology illustrating the various liquid phases occurring with our spray simulation model, revealed a wide arrange of droplet sizes and velocities, thus demonstrating that the mouse cages were exposed to a fast-moving jet and cloud of macro and microdroplets, mimicking a sneeze (**Figures 1B,C**).

Trans-illumination and ImageJ analysis of the textile material (23, 34) used for covering the mouse cages and protect the GF mice from sprayed droplets, revealed a profound reduction (up to 10-fold) of individual and total "pore" area (from 50% of

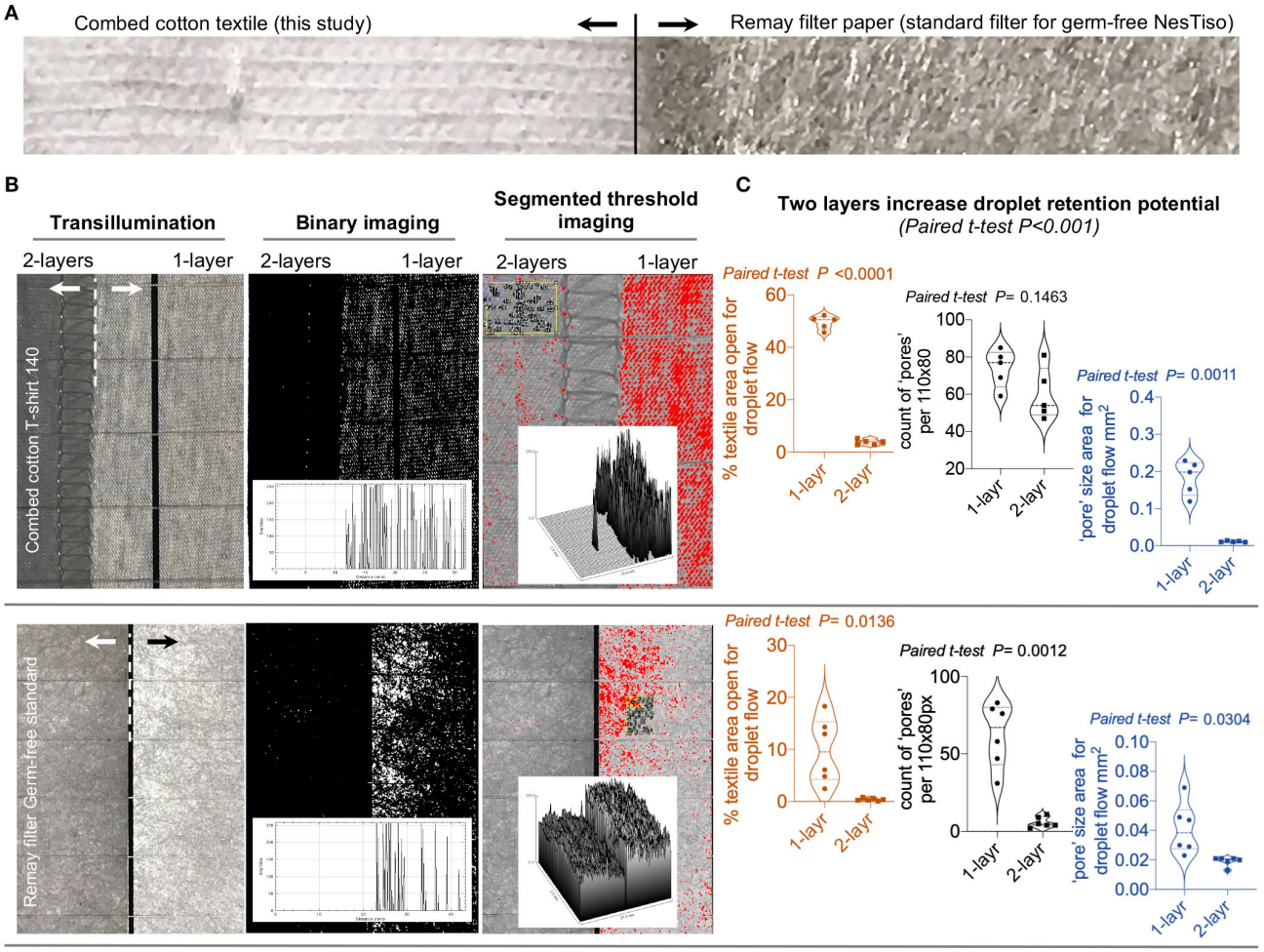


FIGURE 2 | Image analysis with ImageJ to characterize the white light transillumination porosity of 2-layer textiles. **(A)** Photograph of the cotton textile tested with GF mice in this study compared to a Remay™ filter-sheet (Spunbonded Polyester Non-woven Fabrics) used as an analytical "gold-standard" in cage lids for Nested isolation (23) as comparator. **(B)** ImageJ analysis of single and double layers of both cotton and Remay materials. Histogram and surface plots illustrate significant reduction of light passage through textile pores; notice two layers. **(C)** Statistical features of double layers promote increased retention of droplets (pore counts/areas size).

textile area as single layer to 5% as two layers) and counts that allow the flow of light for the cotton textile compared to the "gold-standard" GF-grade Remay filter (**Figures 2A–C**).

Textile data supported the use of two-layer textile barriers for the *in vivo* experiments. In the first phase of the spray experiment with mice, microbiological analysis (fecal culture) of mouse feces before and after two rounds of spray-droplet exposure (2 ml total) at an inoculation dose of 600–1,000 bacterial droplet units per 56.75 cm^2 showed that all GF animals with no textile protection (simulating not wearing a mask) showed signs of microbial contamination within 36 h. In contrast, the GF status of the mice that were covered with the autoclaved textile remained GF after exposure (measured at 120 h), indicating that the textile barrier was extremely effective at retaining bacteria carrying droplets, thus reducing the absolute contamination risk (0/12 vs. 6/6, Fisher's exact, $p < 0.0001$).

The second phase of the experiment testing repeated spray exposure (20 sprays; 10 times as many droplets that initial phase experiment, 20 ml volume of liquid per mouse cage), with 12 GF mice, showed that the textile maintained all animals GF, even after 20 droplet sprays at 60 cm, while mice located at 180 cm became colonized by bacteria-carrying droplets with a single spray (0/8 vs. 4/4, Fisher's exact, $p = 0.002$). Collectively, barriers protected all mice (even with low textile density; heavy vs. light fabric, paired *t*-test, $p = 0.002$) against high droplet doses two or 20 sprays) if the textile fully covered the cage (0/20 vs. 10/10, Fisher's exact, $p < 0.0001$, study power = 1.0).

In the last phase of the spray-experiment, partly covered (95%) cages revealed that, compared to fully unprotected cages, one single dose of droplets at 90 cm of distance (1-spray, ~0.2–0.6 × 10^3 microdroplets) resulted in the bacterial colonization

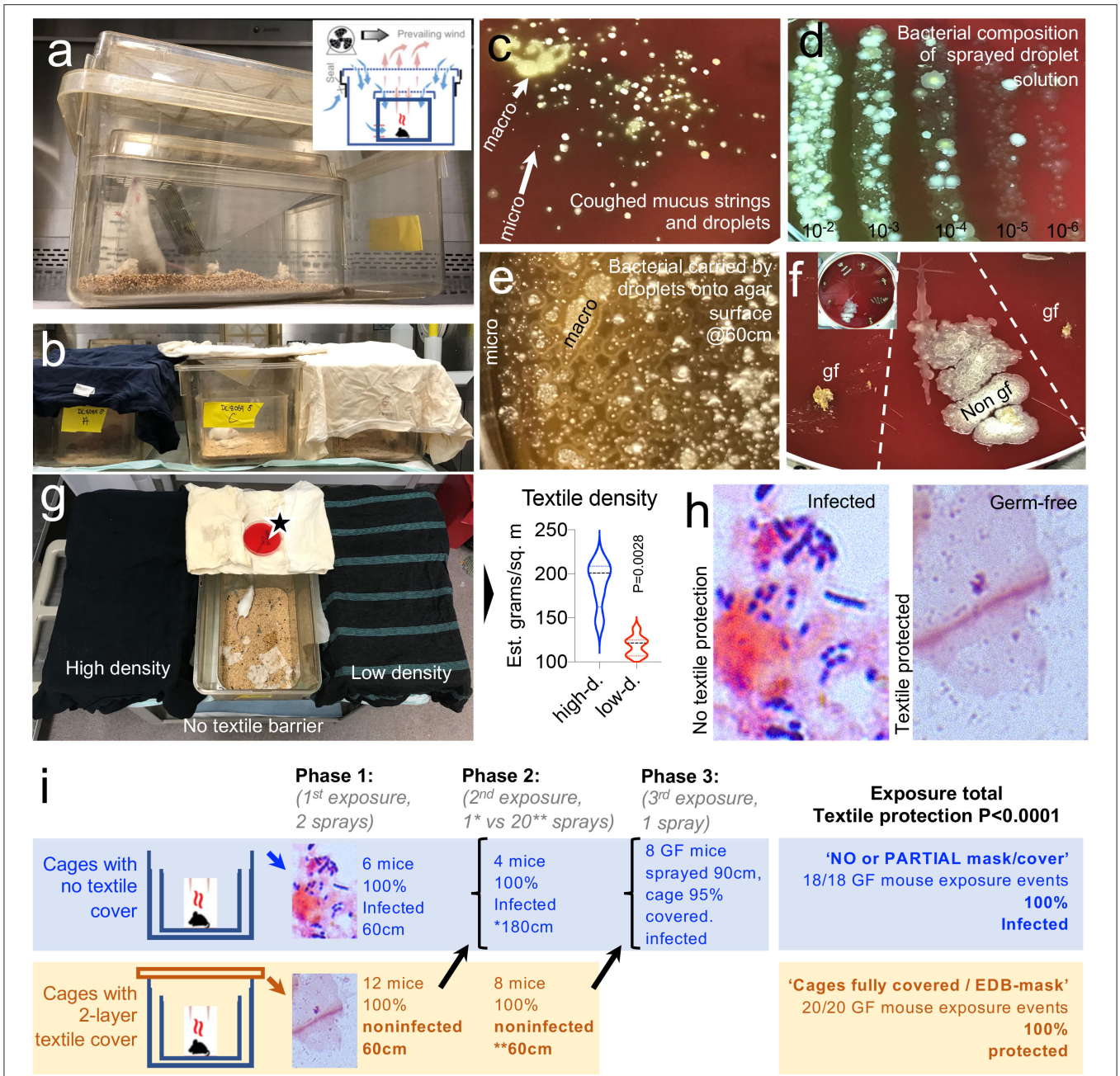


FIGURE 3 | A two-layer textile barrier fully protects germ-free mice from colonization by bacteria in sprayed microdroplets. **(a)** Nested isolation cage housing two-layer system used to raise GF mice (23). **(b)** In this experiment, the two cage lids were replaced by a two-layer textile barrier cover compared with cages without a lid (no cover). Sprayed from 60 to 180 cm distances (see Methods). **(c)** Visualization of bacteria present in cough microdroplets of a healthy adult volunteer. TSA plates, aerobic incubation, 48 h. Note the color, number, size, and relative location and distribution of the bacteria colonies growing from "invisible" microdroplets (CFU) shown as whitish spots on the agar surface. Bacterial growth alters the red color of the fresh non-inoculated agar leading to a brownish discoloring of the petri agars, which is more pronounced as the number of bacterial colonies increase. **(d)** Quantification/visualization of bacterial community in microdroplet solution used to spray GF mice. Parallel lanes plating method (30). **(e)** Visualization of bacteria-contained on macro/microdroplets sprayed on TSA. 21 mm horizontal field. **(f)** Example of fecal culture-negative from mice protected with textiles, which remained GF (gf), and culture-positive from mice not protected with textile (Non-gf), Inset, 20 cm plate, eight samples. **(g)** Two textile densities were tested, but both protected gf mice. Notice the uncovered cage at the center with an open TSA plate located over the cover to verify and quantify the bacteria-carrying microdroplet density that the mice were exposed to. **(h)** Feces, gram stain. See details in **Supplementary Figure 1**. **(i)** Summary of *in vivo* mouse droplet exposure event results. Refer to overview of study design in **Figure 1A** as a referent.

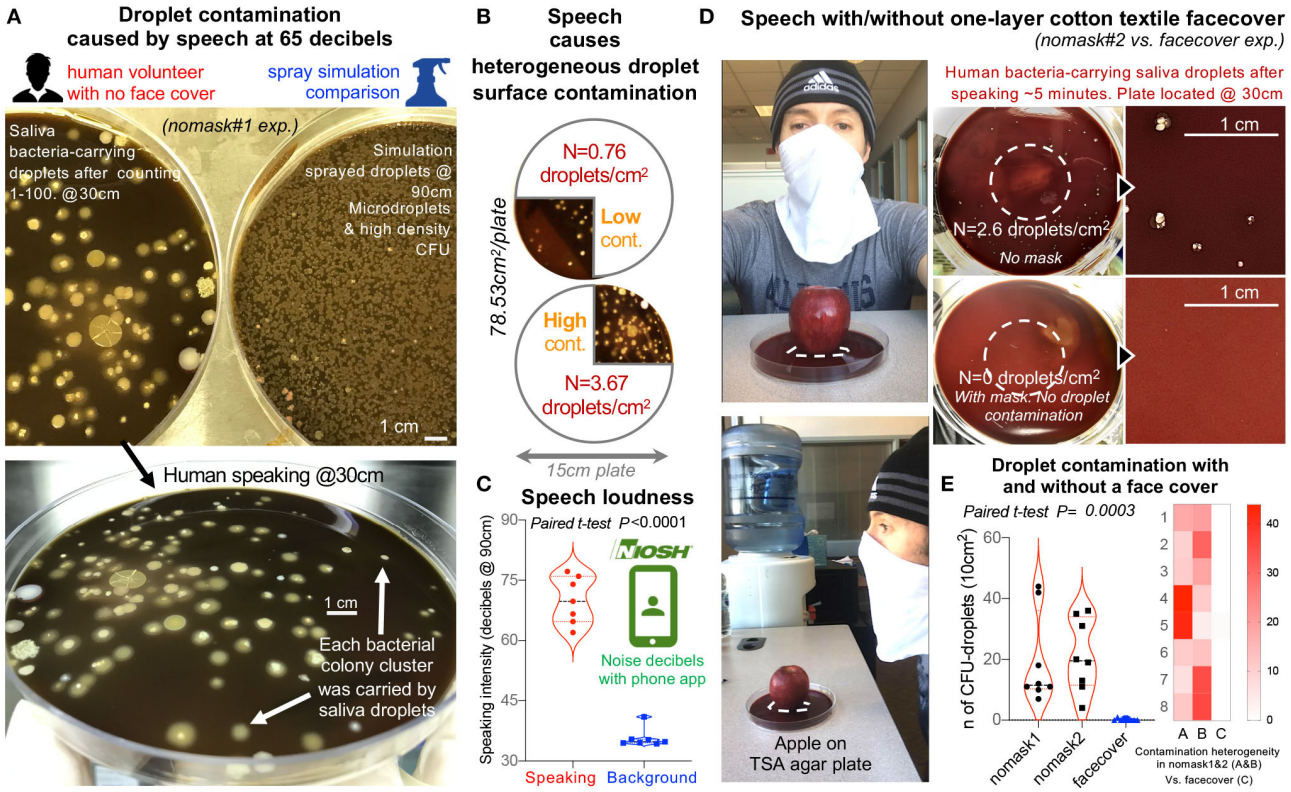


FIGURE 4 | Textile cotton face masks effectively prevent the contamination of the environment with microbial-carrying saliva microdroplets produced by a human volunteer during speech. **(A)** Comparison of bacterial CFU density of plates contaminated by oral droplets during speech (counting from 0 to 100, taking breath every 15–20 numbers, at 30 cm distance from plate) vs. plates with sprayed microdroplets at 90 cm. Large TSA agar plates, 150 mm diameter, and aerobic incubation at 72 h, 37°C. **(B)** Speaking without a face cover causes heterogeneous contamination of environmental surfaces. **(C)** Speech intensity and background noise in decibels (60–75 decibels during speech trials nomask#1 on **(A)** and #2 vs. face cover in **(D)**. Background noise and speech volume intensity were measured in decibels using the Centers for Disease Control and Prevention free phone application NIOSH Sound Level Meter App (https://www.cdc.gov/niosh/topics/noise/app.html). **(D)** Speech experiment repeat by human volunteer with and without a face cover as illustrated, quantified droplet contamination density of between 0.5 and 4 droplets/cm^2 after counting numbers from 1 to 100, and prevention of surface contamination using a single textile layer/barrier. The apple on the agar is to provide a visual and spatial context of the relevance of face covering during speech over food displays/service settings, and to provide the context for a citizen science project we have proposed for adults and children to use the spray simulation model to test the efficacy of face coverings and numerous household textiles available in their homes (see links to websites and educational modules in Spanish, French, English, and Portuguese in Eichler et al., in ref (35). The apple was washed and clean with cloth and ethanol 70% before placed on agar. The dashed lines show area where apple was placed. Note that clean apple yielded no bacteria contamination on agar). **(E)** Summary of droplet contamination for the second speech trial illustrate that, while face covers prevent surface contamination (e.g., 2–6 saliva droplets/cm^2 @30 cm; >2–6 droplets per word based on 78.53 cm^2 plate), the lack of face masks renders the environment heterogeneously contaminated with droplets.

of all (n = 8) mice. Collectively, the number of GF mice that remained GF with a cage fully covered was significantly superior (0/20) compared to the number of mice that were colonized in non-covered or partly covered cages (18/18, Fisher's exact p = 1.14E-06, study power = 1.0; **Figures 3a–i**).

To put the spray experiment in GF mice in human context and perspective, we then tested the ability of the same two-layer cotton textile barrier, used as a face cover, to prevent environmental contamination of an agar surface 10 cm in diameter located at 30 cm with droplets during speech (counting numbers out loud from 1 to 100) conducted within 60–75 decibels. The lack of droplet protection during speech causes the contamination of the environment with bacteria-carrying oral droplets, at heterogeneous densities ranging from 0 to 5 droplets/cm^2 after the short speech trials when measured at 30 cm of distance from the lips. **Figures 4A–E** illustrates that even a single layer of the material used in the experiments above, as spray simulated in another study, was effective at retaining/reducing the risk of environmental contamination by oral/saliva droplets compared to not using a face cover.

DISCUSSION

This study illustrates that GF animals could be used as a functional *in vivo* model to test the effectiveness of textiles as droplet barriers. When protected by two layers of textile (100% combed cotton), all mice were 100% protected from becoming contaminated by the bacteria contained in the

microdroplets. In this context, the study supports that the use of textiles as face covers could be an effective prevention strategy to halt the contamination of the environment with respiratory and saliva/oral microdroplets which may contain known and unknown infectious microorganisms (36, 37).

Although inspired by the current COVID-19 situation and our working model to promote textile face masks and surface covers (21), this study was not intended to address the complex biology of viral infections in humans or as a means to replace long-validated N95 masks, which are fit-tested directly in humans (8). Rather, our study sought to test, *in vivo*, whether two-layer textiles would be effective at preventing the crossing of liquid droplets, mimicking a sneeze. To put the findings into context, our speech trial illustrated that human speech is a constant source of droplet production and contamination. Most importantly, the speech trial illustrated that the textiles tested herein prevented the contamination of the environment with saliva borne microorganisms.

This is the first available study of its kind using GF mice to assess the functional filtration efficiency of liquid micro-droplet material amenable for the fabrication of face masks or covers. Following the pre-print publication of the present study (34), a widely publicized, yet unpublished, study with hamsters indicated that "masks" reduced the contamination of animals with COVID-19 virus by 75% when animals were confined within cages for a week (38). Such preliminary report supports the importance, effectiveness, and value of using surrogate *in vivo* models to study droplets and masks. In future pandemics, the limited access to viruses, or the unwanted need to use such viruses to study face mask effectiveness could be early initiated before the pandemic accelerates using models based on bacterial carrying microdroplets. Toward the future, animal models could be used to further examine the role of droplet barriers in preventing the respiratory transmission of viral particles, for instance, the murine hepatitis *Coronaviridae* virus (39). Although we assessed combed cotton textiles of two densities, studies indicate that most textiles would be effective (21), and beneficial for the control of viral particles (40), or nanoparticles especially if cotton and electrostatic materials are used as a combination in cloth face masks (10, 22).

Of remarkable interest to animal and biomedical research, the textiles herein tested, using the GF-based testing model and NesTiso, were unexpectedly 100% effective at preventing contamination of the mice with the liquid microdroplets. These findings are remarkable because they further support our earlier work in 2018 where we proposed a novel system of breeding and isolation of GF animals using non-pressurized HEPA-filtration anchored methods based on two-layer "nested" isolation (NestIso, nested isolation) (23). In that study, serology conducted at 62 weeks in mice demonstrated that all animals had no titers against 18 highly contagious rodent viruses, including betacoronavirus [see Supplementary Materials in (23)]. Together, findings support the potential to rapidly expanding the research capabilities of using Nested isolation to promote the use of GF animals in disease/microbiological research and assist microbiome research reproducibility (24, 27).

Limitations and Future Directions

The science of textiles is complex, and the study of textiles in particulate/air filtration using *in vitro* systems is becoming a re-emerging field of research since the occurrence of increasingly devastating respiratory pandemics, especially COVID-19 (10, 21, 22, 40). As a novelty, our study was designed to effectively illustrate, as a proof-of-principle, the use of our germ-free mouse housing system/model to examine the filtration potential of any type of materials in an innovative *in vivo* animal system. As such, our findings on the textile specifically used to illustrate the GF model cannot be generalizable to other types of filtration materials, or the number of layers, since each material has their own porosity and hypothetical ability to retain dry and wet droplets or particulates. Future studies could study combinations of materials, practices, or animal genetic lines, or features of the gut microbiota that could modify the susceptibility to droplet-driven infections to tailor current and new potential questions across various fields of science.

Projecting the message from this report into the future via education, along messages from an earlier study from our group on the role of textile barriers reducing droplet contamination distances21, the present studies were used to further support strategies and the need to publicize the relevance of facemasks in the community, especially in schools, as students and workers start returning to highly-populated classrooms and institutions. To promote such efforts, this and our preceding complementary study21 have been used as the foundation to create educational research activities amenable for children and adults, at school and at home, and a citizen science facemask experiment project concurrently launched in multiple languages (Spanish, French. Portuguese and English) to promote COVID-19/coronavirus safety and droplet science awareness (35).

In conclusion, the GF animal protocol herein described is a rapid reliable functional *in vivo* model to test the effectiveness of textiles as droplet barriers or other filtration materials required for infection control or high sterility purposes. Together, the mouse experiment and the speech trial emphasize the benefits of using textiles to enhance the cleanliness of the environment, which can be contaminated by oral-respiratory droplets, regardless of which natural or infectious microorganisms are contained within the droplets.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by IACUC Case Western Reserve University.

AUTHOR CONTRIBUTIONS

AR-P envisioned, planned, executed the experiments, analyzed the data, prepared figures, and wrote the manuscript. MC assisted with experiments. FC commented, revised, and edited the manuscript. All authors approved the final manuscript.

FUNDING

This study was conducted with discretionary funds allocated to AR-P. AR-P received partial support from NIH via grants R21DK118373, entitled Identification of pathogenic bacteria in Crohn's disease, P30DK097948 NIH Silvio O. Conte Cleveland Digestive Diseases Research Core Center, and P01DK091222 (Germ-Free and Gut Microbiome Core) to FC, Case Western Reserve University.

ACKNOWLEDGMENTS

Special thanks to the Animal Resource Center at Case Western Reserve University and Gina Ponzani for assistance with animal husbandry; Dr. Abigail Basson for proofreading and editorial/scientific suggestions; and Drs. Minh Lam, W. John Durfee, and Kristie Brock for assistance with animal utilization protocol and advice relevant to animal housing and study authorization by IACUC.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2020.00504/full#supplementary-material

REFERENCES

1. Asadi S, Wexler AS, Cappa CD, Barreda S, Bouvier NM, Ristenpart WD. Aerosol emission and superemission during human speech increase with voice loudness. *Sci Rep*. (2019) 9:2348. doi: 10.1038/s41598-019-38808-z
2. Dwyer C, Aubrey A. CDC Now Recommends Americans Consider Wearing Cloth Face Coverings In Public. NPR. *Coronavirus crisis*. (2020). Available online at: https://www.npr.org/sections/coronavirus-live-updates/2020/04/03/826219824/president-trump-says-cdc-now-recommends-americans-wear-cloth-masks-in-public2020 (accessed 3 April, 2020).
3. Howard J. Masks May Actually Increase Your Coronavirus Risk if Worn Improperly, Surgeon General Warns. *CNN*. (2020). Available online at: https://www.cnn.com/2020/03/02/health/surgeon-general-coronavirus-masks-risk-trnd/index.html (accessed March 2, 2020).
4. Howard J. WHO Stands by Recommendation to Not Wear Masks if You Are Not Sick or Not Caring for Someone Who Is Sick. *CNN*. (2020). Available online at: https://www.cnn.com/2020/03/30/world/coronavirus-who-masks-recommendation-trnd/index.html (accessed March 31, 2020).
5. BBC. Coronavirus: Trump to Defy "Voluntary" Advice for Americans to Wear Masks. *BBC*. (2020). Available online at: https://www.bbc.com/news/world-us-canada-52161529 (accessed April 4, 2020).
6. Bedford T, Greninger G, Roychoudhury P, Starita LM, Famulare M, Huang ML, et al. Cryptic transmission of SARS-CoV-2 in Washington State. *medRxiv [Preprint]*. (2020) 2020.04.02.20051417. doi: 10.1101/2020.04.02.20051417
7. Mansor S. Data Suggests Many New York City Neighborhoods Hardest Hit by COVID-19 Are Also Low-Income Areas. *Time*. (2020). Available online at: https://time.com/5815820/data-new-york-low-income-neighborhoods-coronavirus/ (accessed April 5, 2020).
8. Howard J, Huang A, Li Z, Tufekci Z, Zdimal V, van der Westhuizen H, et al. Face masks against COVID-19: an evidence review. *Preprints*. (2020) 2020040203. doi: 10.20944/preprints202004.0203.v1
9. MacIntyre CR, Wang Q, Rahman B, Seale H, Ridda I, Gao Z, et al. Efficacy of face masks and respirators in preventing upper respiratory tract bacterial colonization and co-infection in hospital healthcare workers. *Prev Med*. (2014) 62:1–7. doi: 10.1016/j.ypmed.2014.01.015
10. Kilinc FS. A review of isolation gowns in healthcare: fabric and gown properties. *J Eng Fiber Fabr*. (2015) 10:180–90. doi: 10.1177/155892501501000313
11. Shiu EYC, Leung NHL, Cowling BJ. Controversy around airborne versus droplet transmission of respiratory viruses: implication for infection prevention. *Curr Opin Infect Dis*. (2019) 32:372–9. doi: 10.1097/QCO.0000000000000563
12. Tellier R, Li Y, Cowling BJ, Tang JW. Recognition of aerosol transmission of infectious agents: a commentary. *BMC Infect Dis*. (2019) 19:101. doi: 10.1186/s12879-019-3707-y
13. Otter JA, Donskey C, Yezli S, Douthwaite S, Goldenberg SD, Weber DJ. Transmission of SARS and MERS coronaviruses and influenza virus in healthcare settings: the possible role of dry surface contamination. *J Hosp Infect*. (2016) 92:235–50. doi: 10.1016/j.jhin.2015.08.027
14. MacIntyre CR, Chughtai AA. Facemasks for the prevention of infection in healthcare and community settings. *BMJ*. (2015) 350:h694. doi: 10.1136/bmj.h694
15. Cowling BJ, Ip DK, Fang VJ, Suntarattiwong P, Olsen SJ, Levy J, et al. Aerosol transmission is an important mode of influenza A virus spread. *Nat Commun*. (2013) 4:1935. doi: 10.1038/ncomms2922
16. Davies A, Thompson KA, Giri K, Kafatos G, Walker J, Bennett A. Testing the efficacy of homemade masks: would they protect in an influenza pandemic? *Disaster Med Public Health Prep*. (2013) 7:413–8. doi: 10.1017/dmp.2013.43
17. Xie X, Li Y, Sun H, Liu L. Exhaled droplets due to talking and coughing. *J R Soc Interface*. (2009) 6(Suppl.6):S703–14. doi: 10.1098/rsif.2009.0388.focus
18. Chao CYH, Wan MP, Morawska L, Johnson GR, Ristovski ZD, Hargreaves M, et al. Characterization of expiration air jets and droplet size distributions immediately at the mouth opening. *J Aerosol Sci*. (2009) 40:122–33. doi: 10.1016/j.jaerosci.2008.10.003
19. Atkinson MP, Wein LM. Quantifying the routes of transmission for pandemic influenza. *Bull Math Biol*. (2008) 70:820–67. doi: 10.1007/s11538-007-9281-2
20. Loudon RG, Roberts RM. Droplet expulsion from the respiratory tract. *Am Rev Respir Dis*. (1967) 95:435–42.
21. Rodriguez-Palacios A, Cominelli F, Basson A, Pizarro T, Ilic S. Textile masks and surface covers – spray simulation method and a "universal droplet reduction model" against respiratory pandemics. *Front Med*. (2020) 7:260. doi: 10.3389/fmed.2020.00260
22. Konda A, Prakash A, Moss GA, Schmoldt M, Grant GD, Guha S. Aerosol filtration efficiency of common fabrics used in respiratory cloth masks. *ACS Nano*. (2020) 14:6339–47. doi: 10.1021/acsnano.0c03252
23. Rodriguez-Palacios A, Aladyshkina N, Ezeji JC, Erkkila HL, Conger M, Ward J, et al. "Cyclical Bias" in microbiome research revealed by a portable germ-free housing system using nested isolation. *Sci Rep*. (2018) 8:3801. doi: 10.1038/s41598-018-20742-1
24. Basson AR, LaSalla A, Lam G, Kulpins D, Moen EL, Sundrud MS, et al. Artificial microbiome heterogeneity spurs six practical action themes and examples to increase study power-driven reproducibility. *Sci Rep*. (2020) 10:5039. doi: 10.1038/s41598-020-60900-y

25. Rodriguez-Palacios A, Kodani T, Kaydo L, Pietropaoli D, Corridoni D, Howell S, et al. Stereomicroscopic 3D-pattern profiling of murine and human intestinal inflammation reveals unique structural phenotypes. *Nat Commun*. (2015) 6:7577. doi: 10.1038/ncomms8577
26. Research. IfLA. Guide for the Care and Use of Laboratory Animals. 8th ed. Washington, DC: National Academies Press. (2011).
27. Basson A, Cominelli F, Rodriguez-Palacios A. Patterns of "Analytical Irreproducibility" in multimodal diseases. *bioRxiv [Preprint]*. (2020) 2020.03.22.002469. doi: 10.1101/2020.03.22.002469
28. Bourouiba L. Images in clinical medicine. A sneeze. *N Engl J Med*. (2016) 375:e15. doi: 10.1056/NEJMicm1501197
29. Atkinson J, Chartier Y, Pessoa-Silva CL, Jensen P, Li Y, Seto WH. *Natural Ventilation for Infection Control in Health-Care Settings. Annex C Respiratory Droplets*. Geneva: World Health Organization (2009).
30. Rodriguez-Palacios A, Harding A, Menghini P, Himmelman C, Retuerto M, Nickerson KP, et al. The artificial sweetener Splenda promotes gut Proteobacteria, dysbiosis and myeloperoxidase reactivity in Crohn's disease-like ileitis. *Inflam Bowel Dis*. (2018) 24:1005–20. doi: 10.1093/ibd/izy060
31. Kooij S, Sijs R, Denn M, Villermaux E, Bonn D. What determines the drop size in sprays? *Phys Rev X*. 8:031019. doi: 10.1103/PhysRevX.8.031019
32. Han ZY, Weng WG, Huang QY. Characterizations of particle size distribution of the droplets exhaled by sneeze. *J R Soc Interface*. (2013) 10:20130560. doi: 10.1098/rsif.2013.0560
33. Burke W. Why do we sneeze? *Med Hypotheses*. (2012) 78:502–4. doi: 10.1016/j.mehy.2012.01.017
34. Rodriguez-Palacios A, Conger M, Cominelli F. Nonmedical masks in public for respiratory pandemics: droplet retention by two-layer textile barrier fully protects germ-free mice from bacteria in droplets. *bioRxiv [Preprint]*. (2020) 2020.04.06.028688. doi: 10.1101/2020.04.06.028688
35. Eichler S, Hopperton A, Alava J, Pereira A, Amhmed R, Kozlakidis Z, et al. A Citizen science facemask experiment and educational modules to improve coronavirus safety in communities and schools. *Front Med*. (2020) 7:486. doi: 10.3389/fmed.2020.00486
36. CDC. Use of Cloth Face Coverings to Help Slow the Spread of COVID-19. Coronavirus Disease 2019 (COVID-19). (2020). Available online at https://www.cdc.gov/coronavirus/2019-ncov/prevent-getting-sick/diy-cloth-face-coverings.html (accessed April 4, 2020).
37. Adams J. Surgeon General Shows How to Make Your Own Face Covering. (2020). Available online at https://www.youtube.com/watch?v=PI1GxNjAjlw (accessed April 4, 2020).
38. Micu A. ZmeScience. Hamsters Confirm—Face Masks Work Against the Coronavirus. (2020). Available online at https://www.zmescience.com/science/hamster-face-mask-covid-study-15324/ (accessed May 22, 2020).
39. Roth-Cross JK, Bender SJ, Weiss SR. Murine coronavirus mouse hepatitis virus is recognized by MDA5 and induces type I interferon in brain macrophages/microglia. *J Virol*. (2008) 82:9829–38. doi: 10.1128/JVI.01199-08
40. Shimasaki N, Okaue A, Morimoto M, Uchida Y, Koshiba T, Tsunoda K, et al. A multifaceted evaluation on the penetration resistance of protective clothing fabrics against viral liquid drops without pressure. *Biocontrol Sci*. (2020) 25:9–16. doi: 10.4265/bio.25.9

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular and Infection Microbiology

MINI REVIEW
published: 27 August 2020
doi: 10.3389/fcimb.2020.00499

Unraveling the Epidemiology, Geographical Distribution, and Genomic Evolution of Potentially Lethal Coronaviruses (SARS, MERS, and SARS CoV-2)

*Nosheen Masood[2], Saima Shakil Malik[3], Muhammad Naqqi Raja[4], Sumaira Mubarik[1] and Chuanhua Yu[1]**

[1] Department of Epidemiology and Biostatistics, School of Health Sciences, Wuhan University, Wuhan, China, [2] Department of Biotechnology, Fatima Jinnah Women University, Rawalpindi, Pakistan, [3] Department of Zoology, University of Gujrat, Gujrat, Pakistan, [4] Oil and Gas Development Company Limited, Islamabad, Pakistan

OPEN ACCESS

Edited by:
Donghoon Chung, University of Louisville, United States

Reviewed by:
Alessia Zamborlini, Université Paris-Sud, France
Maleewan Kitcharoensakkul, Washington University in St. Louis, United States

***Correspondence:**
Chuanhua Yu
yuchua@whu.edu.cn

Specialty section:
This article was submitted to Virus and Host, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: 27 March 2020
Accepted: 11 August 2020
Published: 27 August 2020

Citation:
Masood N, Malik SS, Raja MN, Mubarik S and Yu C (2020) Unraveling the Epidemiology, Geographical Distribution, and Genomic Evolution of Potentially Lethal Coronaviruses (SARS, MERS, and SARS CoV-2). Front. Cell. Infect. Microbiol. 10:499. doi: 10.3389/fcimb.2020.00499

SARS CoV appeared in 2003 in China, transmitted from bats to humans via eating infected animals. It affected 8,096 humans with a death rate of 11% affecting 21 countries. The receptor binding domain (RBD) in S protein of this virus gets attached with the ACE2 receptors present on human cells. MERS CoV was first reported in 2012 in Middle East, originated from bat and transmitted to humans through camels. MERS CoV has a fatality rate of 35% and last case reported was in 2017 making a total of 1,879 cases worldwide. DPP4 expressed on human cells is the main attaching site for RBD in S protein of MERS CoV. Folding of RBD plays a crucial role in its pathogenesis. Virus causing COVID-19 was named as SARS CoV-2 due its homology with SARS CoV that emerged in 2003. It has become a pandemic affecting nearly 200 countries in just 3 months' time with a death rate of 2–3% currently. The new virus is fast spreading, but it utilizes the same RBD and ACE2 receptors along with furin present in human cells. The lessons learned from the SARS and MERS epidemics are the best social weapons to face and fight against this novel global threat.

Keywords: SARS CoV, MERS CoV, SARS CoV-2, COVID-19, ACE2, DPP4

INTRODUCTION

Formerly, six different coronaviruses (CoVs) have been known as disease causing among humans in which two alpha-CoVs (HCoV-NL63 and HCoV-229E) and two beta-CoVs (HCoV-OC43 and HCoV-HKU1) have low pathogenicity (Cui et al., 2019). Whereas two already known beta-CoVs; severe acute respiratory syndrome (SARS) and Middle East respiratory syndrome coronavirus (MERS) caused potentially fatal and extremely severe respiratory tract infections (Wang et al., 2013). In December 2019, a novel CoV named COVID-19 or SARS CoV-2 emerged in Wuhan city of Hubei province, China and transmitted to almost 192 countries around the globe in just 3 months with 3 435,036 cases and 19,607 deaths till 25th March, 2020. Therefore, there is a need to understand the main mechanism that underlie in this enormous spreading capability of SARS

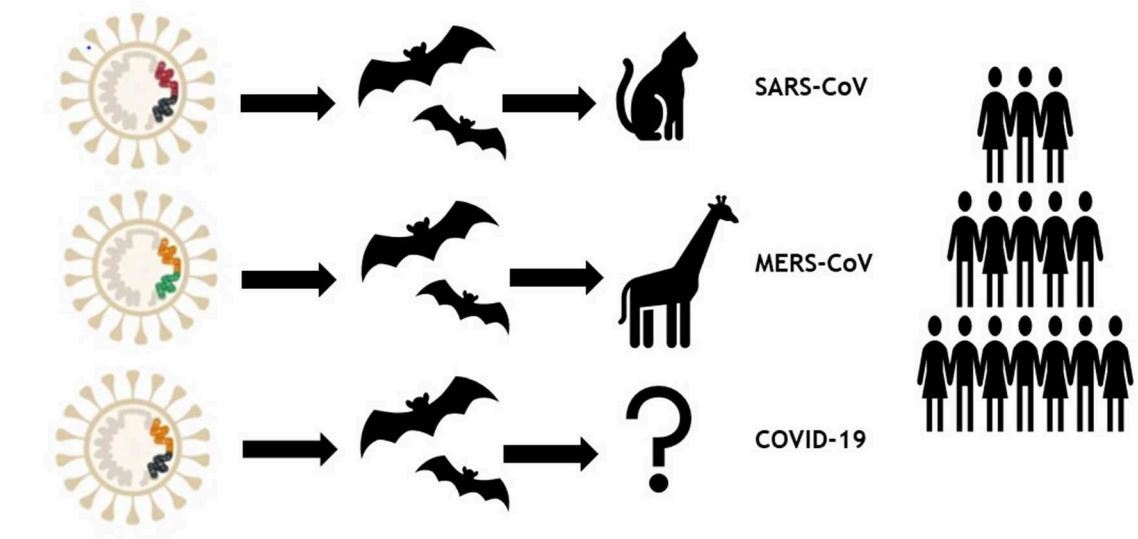


FIGURE 1 | Genetically modified coronaviruses and their transmission to human.

CoV-2 compared with other viruses of same group (**Figure 1**). The present study may help the researchers identifying the main route of vaccine success by getting a genomic, geographic, and epidemiologic comparison among SARS CoV, MERS CoV, and SARS CoV-2.

SARS COV

Epidemiology

SARS CoV originated in bats in China and transferred to humans via civet cats (Low, 2004). It has many reservoirs among these are animals, human, and laboratories. As SARS CoV have been isolated from raccoon dogs, ferrets, and Himalayan palm civets and these animals are consumed by humans living in China. Its outbreak started in 2003 however no case of SARS CoV is reported after 2004 (Donnelly et al., 2003). The most affected country was China with 5,000 plus incidence rate and 349 deaths followed by Hong Kong with 1,500 plus cases and 299 deaths. The worst hit areas of the world include China, Hong Kong, Taiwan, Canada, Singapore, Vietnam, US, and Philippines. Rest of the 21 countries had <10 reported cases of SARS CoV (World Health Organization, 2003). Data for SARS CoV transmission, incidence and geographic information was retrieved from World Health Organization available at: https://www.who.int/csr/sars/en/ and presented on the world map using MapChart (**Figure 2A**). It was reported by Low (2004) that SARS CoV will not reappear due to limited reservoir of virus and isolation and precaution measures taken. Once it is gone it will not return. SARS is an atypical pneumonia that first emerged in Guangdong Province of China in 2003 and later spread in many countries. The mortality rate of SARS is about 11% with increased risk in older patients above 60 years of age (Choi et al., 2003).

SARS CoV Genome Structure and Mode of Action

Later, the causative agent of this disease was identified as a virus of corona family named as SARS-CoV. Coronaviruses have large, positive-stranded, RNA genomes ranging from 27 to 31 kb in size and among them SARS-CoV has RNA genome of ∼30 kb (He et al., 2003). It consists of 5′ and 3′ UTR regions flanking 14 open reading frames. The 5′ untranslated region is of 265 bp whereas 3′ end has 342 bp. In all the families of coronavirus the ORFs 1a, 1b, 2, 4, 5, 6, and 9a are conserved. Once the SARS CoV is inside a suitable host ORFs 1a and 1b (that is approximately first two third of genome) start translation of two large polyprotiens (pp1a and pp1ab that are 486 and 790 kDa, respectively) that is cleaved by papain like proteinase 2 and 3C like proteinase encoded by virus in to non-structural proteins known as coronavirus replication complex containing 16 mature replicase proteins (Snijder et al., 2003). The CoV nsps comprise of proteins having enzymatic activities consistent with roles in RNA synthesis or modification, including: RNA-dependent RNA polymerase (RdRp; nsp12), RNA primase (nsp8), helicase-NTPase (nsp13), exoribonuclease (ExoN; nsp14), endoribonuclease (EndoU; nsp15), RNA 2′-O-methyltransferase (MT; nsp16), and RNA cap N7-methyltransferase activity (nsp14). Structure of the virus revealed three conserved motifs I to III of the DEDD superfamily in nsp14 with a zinc finger domain and zinc-coordinating residues (Eckerle et al., 2010). These proteins are responsible for replication of virus as well as synthesis of nested sets of subgenomic mRNAs that transcribe all the remaining ORFs. Discontinuous nested sets are formed due to transcription regulating sequences (TRSs) at the 5′ end. The proteins formed from the last one third portion of genome are of four different types namely structural spike protein (S), membrane proteins

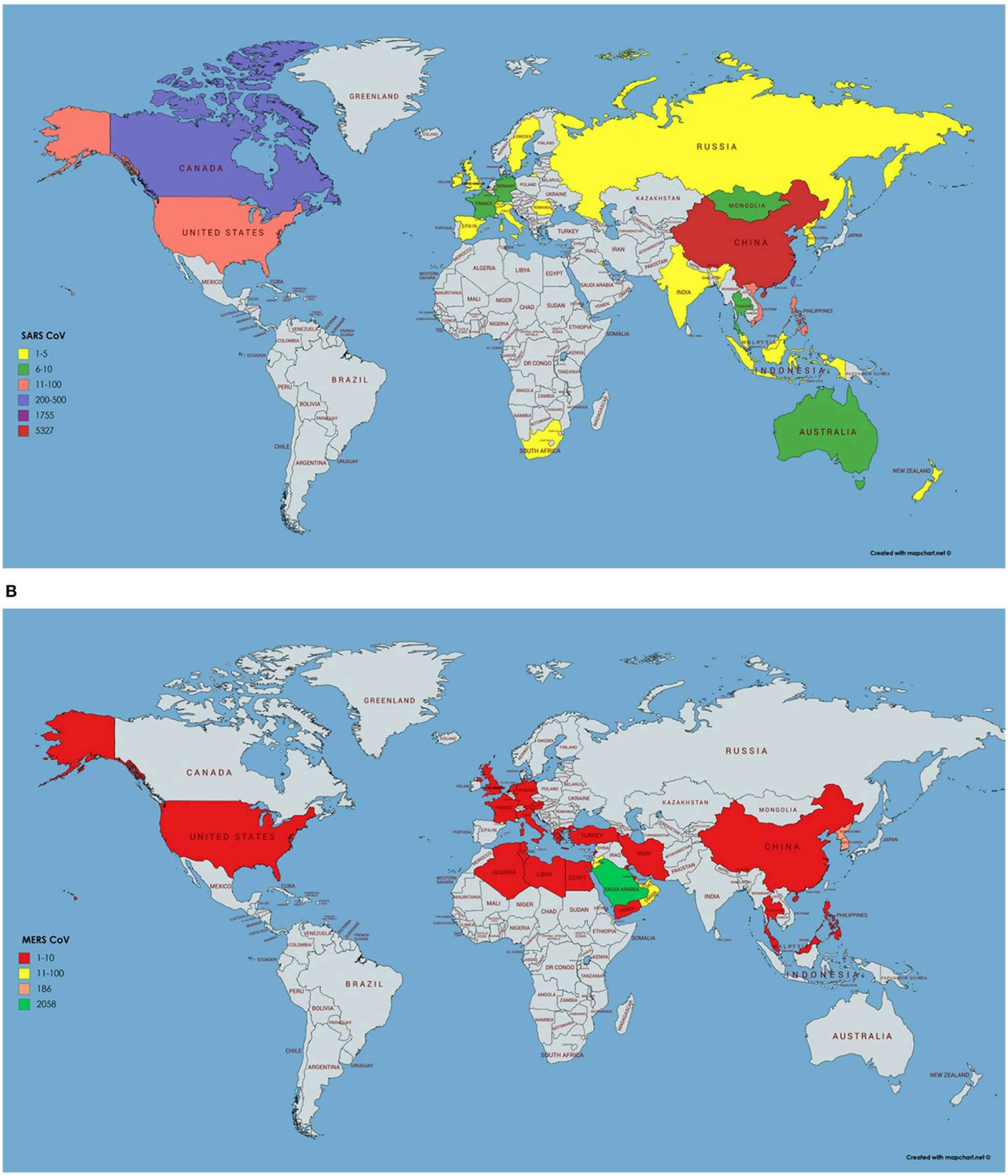


FIGURE 2 | Continued

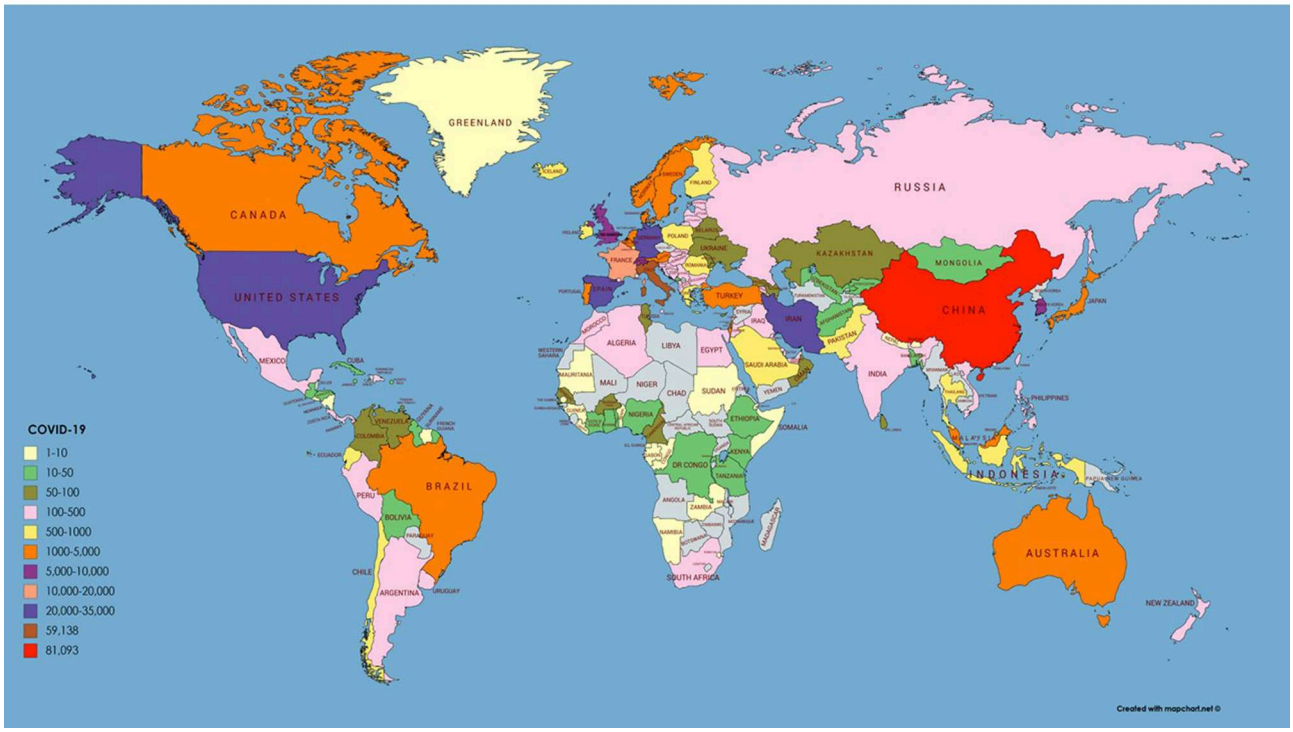


FIGURE 2 | (A) Number of infected cases and Geographical distribution of SARS CoV around the globe. Key is provided in the map. **(B)** Number of infected cases and Geographical distribution of MERS CoV around the globe. **(C)** Number of infected cases and Geographical distribution of SARS CoV-2 around the globe as per 23rd March 2020.

(M), envelope proteins (E), and nucleocapsid proteins (N). First two proteins are directed via endoplasmic reticulum and Golgi compartments. The RNA protein complex then joins with the M protein and nucleocapsid particle buds into the endoplasmic reticulum followed by Golgi apparatus and migrates outside the cell by exocytosis. Along with these proteins a large number of sets of accessory proteins are also formed and their sequence vary among the coronavirus family and gives it a unique importance of having large polyprotein (Baranov et al., 2005). The genes encoded by ORF 3a, 3b, 6, 7a, 7b, 8a, 8, and 9b are not found in other coronavirus families. This virus identifies the host through the proteins that are attached in the S protein. Many phylogenetic analysis have been carried out on SARS CoV genome and found it to be an early split off lineage from coronavirus group II (Thiel et al., 2003).

SARS CoV does not identify the previously known coronavirus family infecting receptors but angiotensin converting enzyme 2 (ACE2) is the target receptor of human cells (Li et al., 2003). Usually the S protein of corona virus is cleaved into two subunits S1 and S2 but in case of SARS CoV an uncleaved type I transmembrane S protein is found with S1 and S2 subunit homology (Xiao et al., 2003). The 193 aminoacid fragment of S protein is involved in infection and specifically its 318–510 residues bind with ACE2 receptor (Jauregui et al., 2013). A receptor binding domain (RBD) on S helps in the binding of virus with peptidase domain of human ACE2. The ultrastructure of RBD showed that it is structurally modified and is concave surface cradles well with the N terminal of peptidase resulting in providing attachment site for SARS CoV (He et al., 2004; Li et al., 2005).

MERS COV

Epidemiology

In 2012, Middle East respiratory syndrome coronavirus (MERS CoV), was first reported among humans in the Middle East, and then transmitted to numerous European countries. Emergence of MERS-CoV involved dromedary camels, as, CoV strains isolated from camels were almost identical to the human CoVs (Haagmans et al., 2014). Laboratory confirmed cases of MERS-CoV were reported in Saudi Arabia, Jordan, Qatar, United Arab Emirates, France, United Kingdom, Germany, Tunisia, and Italy (Bermingham et al., 2012; Zaki et al., 2012; de Groot et al., 2013). All countries were linked to the Middle East, since the infected cases either traveled or had been in close contact with people that recently traveled to that region. A substantial number of the infected patients (∼50%) had developed severe respiratory illness and other clinical symptoms quite like those observed during SARS outbreak in 2003 (Hui et al., 2014; Zumla et al.,

2015). Precisely, epidemiological studies had suggested a human-to-human transmission of MERS, heading toward a pandemic (van Boheemen et al., 2012). As of 16th Jan. 2017, a total 1,879 MERS CoV cases with 659 deaths were reported by WHO, worldwide. The fatality rate in infected cases (35%) is much greater than that of SARS which was found to be 11%. The SARS epidemic exhibited an increased estimated reproductive number, peaked, declined, and finished in 8 months whereas MERS has less reproductive number and absurdly continued with mostly sporadic pattern for more than 4 years. MERS CoV transmission, incidence and geographic information was retrieved from WHO repositories available at https://www.who.int/emergencies/mers-cov/en/ (**Figure 2B**).

MERS CoV Genome Structure and Mode of Action

MERS CoV is a zoonotic disease, part of lineage C of betacoronavirus genus, intimately linked with Pipistrellus bat coronavirus (HKU5) and Tylonycteris bat coronavirus (HKU4) determined from the genetic and phylogenetic analysis, while exact reservoir and source of MERS CoV remains ambiguous (Woo et al., 2012). MERS CoV just like other members of its class exploits a huge surface spike glycoprotein (S) to interact with the target cell and entrance into it (Jiang et al., 2013). This glycoprotein comprises of four different domains among them first one is a globular S1 domain at the N – terminal, afterwards membrane proximal S2, a transmembrane, and an intracellular domain is present (Du et al., 2009; Wang et al., 2013). S1 domain contains all the necessary elements for cellular tropism and interplay with the target cell whereas S2 domain entails membrane fusion mediators (Millet and Whittaker, 2015, 2018). Dipeptidyl peptidase−4 (DPP4) also termed as CD26 acts as a cellular receptor for MERS-CoV, identified by copurification with S1 domain of this deadly virus (Doulkeridou, 2013; Wirblich et al., 2017). None of the structural or sequence similarities of DPP4/CD26 were shared with formerly reported human coronavirus receptors like ACE2 and HCoV-NL63/aminopeptidase N (APN) for SARS-CoV and HCoV-229E, respectively (Forni et al., 2017; Wan et al., 2020; Zhou et al., 2020). DPP4/CD26 is also expressed on surface of various cell types such as those endowed with ectopeptidase activity and resides in human airways just like APN and ACE2 (Lu et al., 2013; Walls et al., 2016). However, this enzymatic functioning is not required for the viral entry into the host. Sequencing and modeling experiments of multidimensional S glycoprotein from numerous human CoVs has exhibited a potent receptor—binding domain (RBD) of MERS CoV (McKimm-Breschkin et al., 2018). But, less homology among S glycoprotein sequences and interaction mechanisms with the definite cell surface receptors, manifests significant changeability in structural attributes amongst corresponding RBD receptor pairs (Liu et al., 2020). The DPP4/CD26 extracellular domain comprises of N–terminal 8–bladed–β-propeller domain (each consists of 4 antiparallel β strands) with a C–terminal α/β-hydrolase domain. The DPP4/CD26 binds only with 4 and 5 number blades in order to contract MERS RBD and no binding interaction was observed for other blades conceivably because of shape and charge complementarities. Explicitly, the outer surface of blades 4 and 5 in DPP4/CD26 β-propeller domain contains 3 positively charged residues including K267, R317, and R336 which interact with 4 negatively charged residues i.e., D510, D537, D539, and E536 on the RBD surface (Lu et al., 2013; Wang et al., 2013). Additionally, the contact/enzymatic site was found to be far away from the hydrolase domain elucidated by adding DPP4/CD26 inhibitors (vildagliptin, sitagliptin, and saxagliptin) which does not block the entrance of MERS CoV following the structural pattern of ACE2 binding with SARS CoV receptor binding domain (Al-Tawfiq and Memish, 2017; Takagaki et al., 2017; Shao et al., 2020). In addition to potential differences in ACE2 and DPP4/CD26 expression levels and distribution in various tissues their structural modifications are anticipated to play crucial role in *in vivo* cell tropism verification along with pathogenesis of SARS and MERS coronaviruses (Bradley and Bryan, 2019; Jaimes et al., 2020). Few sequence alterations in the contact residues of DPP4/CD26 from different mammals diverged researcher's attention toward the exploration of cell susceptibility and MERS-CoV host range (Lau et al., 2018; Letko et al., 2018). Vaccination is the only beneficial measure to fight against viral infection and its transmission. Many antibodies exhibit neutralization activity by targeting receptor binding domain and thus disrupting the virus—receptor interaction. Hence, accurately folded RBD could serve as an ideal immunogen for vaccination (Modjarrad, 2016; Al-Amri et al., 2017).

SARS CoV-2

In December 2019, an outbreak of pneumonia cases occurred due to novel β-coronavirus resembling SARS in Wuhan, Hubei province, China named as COVID-19 by WHO on 12 January 2020 (Zhou et al., 2020). As of March 25, 2020, a total of 81,218 SARS CoV-2 cases have been confirmed in China including 73,650 recovered and 3,281 deaths. Recent literature has shown 2.2 reproduction number of SARS CoV-2 which can reach up to 6.5 and spreading progressively by human–to–human transmission in 192 countries and territories. SARS CoV-2 has 96.2% sequence similarity with a bat CoV, RaTG13, and shared 79.5% similarity with SARS CoV, that's why present virus is also named as SARS CoV-2 by Coronavirus Study Group of the International Committee on February 11, 2020 (Liu et al., 2020). Therefore, based on evolutionary, genomic, and proteomic investigations bat has been suspected as natural host of SARS CoV-2 and it might be transmitted from bats to the humans through some mysterious intermediate hosts.

Epidemiology, Transmission, and Reservoirs

On December 12, 2019, SARS CoV-2 an epidemic of unidentified respiratory tract infection exploded first in Wuhan a city of province Hubei, China probably linked to a seafood market. However, no evidence is available yet of their seafood market origin and bats are suggested to be their potential reservoirs, confirmed by the genome sequencing (Giovanetti et al., 2020; Liu et al., 2020; Paraskevis et al., 2020). Additionally, phylogenetic analysis and protein sequence alignment presented

that analogous ACE2 receptor residues were found in many other species, which explain the prospects of substitutive intermediate hosts like snakes, turtles, and pangolin (Banerjee et al., 2019; Zhou et al., 2020). People who have traveled to Wuhan or encounter the individuals who visited Wuhan have developed this viral infection and transmitted it all over the world. Wuhan Spring Festival would be a possible reason behind this much fast transmission of SARS CoV-2 around the globe as thousands of people have attended it (Wang et al., 2020). As per March 25, 2020, 69,176 COVID-19 cases and 6,820 deaths were recorded in Italy. In the United States, 54,968 new cases with 784 deaths were recorded on March 25, 2020 and the situation is getting worse all over the world except in China (COVID-19 CORONAVIRUS PANDEMIC, 2020). As per March 23, 2020, epidemiology/incidence and transmission of SARS CoV-2 around the globe is shown in **Figure 2C**.

Genome Structure and Mode of Action

SARS CoV-2 genome (29.9 kb) was isolated from a patient admitted due to severe respiratory syndrome at Wuhan and working in a seafood market (Wu F. et al., 2020). Whereas RNA genomes of SARS and MERS CoVs were of 27.9 and 30.1 kb size, respectively (de Wit et al., 2016). Variable number of ORFs (6–11) are present in the COVID-19 genome (Song et al., 2019). Most of the viral RNA portion resides in the first ORF, encoding 16 non-structural proteins, translating 2 polyproteins (pp1a and pp1ab) whereas, rest of the ORFs encodes structural and accessory protein. The remaining part of the virus genome encodes for four crucial structural proteins such as spike glycoprotein (S), matrix protein (M), Envelope protein (E), and nucleocapsid protein (N) together with various accessory proteins responsible for interfering with host immune response (Cui et al., 2019). In comparison with previously known pathogenic CoVs genome, SARS, and MERS, COVID-19 shares more sequence similarity with SARS like bat CoVs. As, most of the genome encoded COVID-19 proteins are like SARS CoVs with certain differences. No amino acid alterations were found in the nucleocapsid (NSP7 and NSP13), matrix, accessory (8b and p6), or envelope proteins. However, at the protein level, few substitutions were observed in nucleocapsid (NSP2 and NSP3), spike protein and RBD (Wu A. et al., 2020). Nucleocapsid (NSP2 and NSP3) protein alterations play significant role in differentiation mechanism and infectious capability of COVID-19 (Angeletti et al., 2020). This triggers researchers to investigate the host tropism and transmission differences among SARS-CoV and SARS CoV-2 or explore potential therapeutic targets (Zhang et al., 2020). It was confirmed that COVID-19 utilizes similar cellular entry receptor ACE2 just like SARS CoV. The S glycoprotein of CoVs binds with ACE2 receptor on human cells surface leading to its entry into the cell, and various approaches are in progress to explore and inhibit this binding. Moreover, it was found that SARS CoV-2 genotype mutated in different patients in China (Tang et al., 2020), emphasizing in-depth investigations of epidemic and virulence.

One of the recently published articles reported the structural basis of COVID-19 interaction with ACE2. The trimeric COVID-19 S1 spike binds with the PD domain of ACE2 and cause cleavage of ACE2 C-terminal segment (residues 697–716) by the transmembrane protease serine 2 (TMPRSS2) enhances the S-protein-driven viral entry. They have compared the 805 amino acid residues of the 10 human ACE2 proteins and the four different ACE2 isoforms available via GeneBank using Clustal Omega multiple sequence alignment, and found 100% identity between the complete ACE2 sequences and the isoforms corresponded to a deletion in the CLD domain, or transmembrane domain truncation (Hoffmann et al., 2020). Researchers are still struggling to explore the role of these isoforms in SARS CoV-2 infection and COVID-19 outcome. Cao et al., demonstrated 32 ACE2 variants in different populations among which seven are hotspot variants including Lys26Arg, Asn638Ser, Ile486Val, Ala627Val, Ser692Pro, Leu731Ile/Phe, and Asn720Asp. This evidence leads to the possibility that some of the individuals could be less susceptible to SARS CoV-2 infection than others (Cao et al., 2020).

CONCLUSION

Collectively genomic, evolutionary, pathogenic, and receptor binding data elucidated that SARS CoV, MERS CoV, and SARS CoV-2 most probably originated in bats via sequential recombination's of SARS-CoVs. Genetic alterations in ORFs and S glycoprotein lead to their spread in many other animals who transmitted these deadly viruses to humans leading to human–to–human transmission. Currently, no treatment is available, and researchers are struggling to find potential therapeutics by targeting RBD. In addition, we suggest sustaining barriers between human society and natural reservoirs in order to prevent zoonotic diseases. Knowledge about SARS CoV-2 is increasing with every single day and there is still much more to know specifically about its epidemiological, genomic and immunological features responsible for spread on a pandemic level. The lessons learned from the SARS and MERS epidemics are the best social weapons we must face this novel global threat.

AUTHOR CONTRIBUTIONS

NM: concept and write up. SSM: concept, data collection, and write up. MR: geographical distribution and map construction. SM: epidemiology and data collection. CY: concept, proof reading, and guidance. All authors contributed to the article and approved the submitted version.

FUNDING

This project was supported by the National Key Research and Development Program of China (Grant nos. 2017YFC1200502 and 2018YFC1315302), the National Natural Science Foundation of China (Grant no. 81773552) and the Fundamental Research Funds for the Central Universities (Grant no. 2020YJ066).

REFERENCES

Al-Amri, S. S., Abbas, A. T., Siddiq, L. A., Alghamdi, A., Sanki, M. A., Al-Muhanna, M. K., et al. (2017). Immunogenicity of candidate MERS-CoV DNA vaccines based on the spike protein. *Sci. Rep.* 7:44875. doi: 10.1038/srep44875

Al-Tawfiq, J. A., and Memish, Z. A. (2017). Update on therapeutic options for Middle East respiratory syndrome coronavirus (MERS-CoV). *Expert Rev. Anti Infect. Ther.* 15, 269–275. doi: 10.1080/14787210.2017.1271712

Angeletti, S., Benvenuto, D., Bianchi, M., Giovanetti, M., Pascarella, S., and Ciccozzi, M. (2020). COVID-2019: the role of the nsp2 and nsp3 in its pathogenesis. *J. Med. Virol.* 92, 584–588. doi: 10.1002/jmv.25719

Banerjee, A., Kulcsar, K., Misra, V., Frieman, M., and Mossman, K. (2019). Bats and coronaviruses. *Viruses* 11:41. doi: 10.3390/v11010041

Baranov, P. V., Henderson, C. M., Anderson, C. B., Gesteland, R. F., Atkins, J. F., and Howard, M. T. (2005). Programmed ribosomal frameshifting in decoding the SARS-CoV genome. *Virology* 332, 498–510. doi: 10.1016/j.virol.2004.11.038

Bermingham, A., Chand, M., Brown, C., Aarons, E., Tong, C., Langrish, C., et al. (2012). Severe respiratory illness caused by a novel coronavirus, in a patient transferred to the United Kingdom from the Middle East, September 2012. *Euro. Surveill.* 17:20290. doi: 10.2807/ese.17.40.20290-en

Bradley, B.T., and Bryan, A. (2019). Emerging respiratory infections: the infectious disease pathology of sars, mers, pandemic influenza, and legionella. *Semin. Diagn. Pathol.* 36, 152–159. doi: 10.1053/j.semdp.2019.04.006

Cao, Y., Li, L., Feng, Z., Wan, S., Huang, P., Sun, X., et al. (2020). Comparative genetic analysis of the novel coronavirus (2019-nCoV/SARS-CoV-2) receptor ACE2 in different populations. *Cell Discov.* 6, 1–4. doi: 10.1038/s41421-020-0147-1

Choi, K. W., Chau, T. N., Tsang, O., Tso, E., Chiu, M. C., Tong, W. L., et al. (2003). Outcomes and prognostic factors in 267 patients with severe acute respiratory syndrome in Hong Kong. *Ann. Intern. Med.* 139, 715–723. doi: 10.7326/0003-4819-139-9-200311040-00005

COVID-19 CORONAVIRUS PANDEMIC (2020). Available online at: https://www.worldometers.info/coronavirus/ (accessed March 25, 2020).

Cui, J., Li, F., and Shi, Z.-L. (2019). Origin and evolution of pathogenic coronaviruses. *Nat. Rev. Microbiol.* 17, 181–192. doi: 10.1038/s41579-018-0118-9

de Groot, R. J., Baker, S. C., Baric, R. S., Brown, C. S., Drosten, C., Enjuanes, L., et al. (2013). Commentary: Middle East respiratory syndrome coronavirus (MERS-CoV): announcement of the Coronavirus Study Group. *J. Virol.* 87, 7790–7792. doi: 10.1128/JVI.01244-13

de Wit, E., van Doremalen, N., Falzarano, D., and Munster, V. J. (2016). SARS and MERS: recent insights into emerging coronaviruses. *Nat. Rev. Microbiol.* 14:523. doi: 10.1038/nrmicro.2016.81

Donnelly, C. A., Ghani, A. C., Leung, G. M., Hedley, A. J., Fraser, C., Riley, S., et al. (2003). Epidemiological determinants of spread of causal agent of severe acute respiratory syndrome in Hong Kong. *Lancet* 361, 1761–1766. doi: 10.1016/S0140-6736(03)13410-1

Doulkeridou, S. (2013). Middle East respiratory syndrome coronavirus (MERS-CoV): a review. *Rev. Francoph Lab.* 14:455. doi: 10.1016/S1773-035X(13)72154-3

Du, L., He, Y., Zhou, Y., Liu, S., Zheng, B.-J., and Jiang, S. (2009). The spike protein of SARS-CoV—a target for vaccine and therapeutic development. *Nat. Rev. Microbiol.* 7, 226–236. doi: 10.1038/nrmicro2090

Eckerle, L. D., Becker, M. M., Halpin, R. A., Li, K., Venter, E., Lu, X., et al. (2010). Infidelity of SARS-CoV Nsp14-exonuclease mutant virus replication is revealed by complete genome sequencing. *PLoS Pathog.* 6:e1000896. doi: 10.1371/journal.ppat.1000896

Forni, D., Cagliani, R., Clerici, M., and Sironi, M. (2017). Molecular evolution of human coronavirus genomes. *Trends Microbiol.* 25, 35–48. doi: 10.1016/j.tim.2016.09.001

Giovanetti, M., Benvenuto, D., Angeletti, S., and Ciccozzi, M. (2020). The first two cases of 2019-nCoV in Italy: where they come from? *J. Med. Virol.* 92, 518–521. doi: 10.1002/jmv.25699

Haagmans, B. L., Al Dhahiry, S. H., Reusken, C. B., Raj, V. S., Galiano, M., Myers, R., et al. (2014). Middle East respiratory syndrome coronavirus in dromedary camels: an outbreak investigation. *Lancet Infect. Dis.* 14, 140–145. doi: 10.1016/S1473-3099(13)70690-X

He, R., Leeson, A., Andonov, A., Li, Y., Bastien, N., Cao, J., et al. (2003). Activation of AP-1 signal transduction pathway by SARS coronavirus nucleocapsid protein. *Biochem. Biophys. Res. Commun.* 311, 870–876. doi: 10.1016/j.bbrc.2003.10.075

He, Y., Zhou, Y., Liu, S., Kou, Z., Li, W., Farzan, M., et al. (2004). Receptor-binding domain of SARS-CoV spike protein induces highly potent neutralizing antibodies: implication for developing subunit vaccine. *Biochem. Biophys. Res. Commun.* 324, 773–781. doi: 10.1016/j.bbrc.2004.09.106

Hoffmann, M., Kleine-Weber, H., Schroeder, S., Krüger, N., Herrler, T., Erichsen, S., et al. (2020). SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor. *Cell* 181, 271–280. doi: 10.1016/j.cell.2020.02.052

Hui, D. S., Memish, Z. A., and Zumla, A. (2014). Severe acute respiratory syndrome vs. the Middle East respiratory syndrome. *Curr. Opin. Pulmonary Med.* 20, 233–241. doi: 10.1097/MCP.0000000000000046

Jaimes, J. A., Andre, N. M., Millet, J. K., and Whittaker, G. R. (2020). Structural modeling of 2019-novel coronavirus (nCoV) spike protein reveals a proteolytically-sensitive activation loop as a distinguishing feature compared to SARS-CoV and related SARS-like coronaviruses. *J. Mol. Biol.* doi: 10.1101/2020.02.10.942185. [Epub ahead of print].

Jauregui, A. R., Savalia, D., Lowry, V. K., Farrell, C. M., and Wathelet, M. G. (2013). Identification of residues of SARS-CoV nsp1 that differentially affect inhibition of gene expression and antiviral signaling. *PLoS ONE* 8:e62416. doi: 10.1371/journal.pone.0062416

Jiang, S., Lu, L., Du, L., and Debnath, A. K. (2013). A predicted receptor-binding and critical neutralizing domain in S protein of the novel human coronavirus HCoV-EMC. *J. Infect.* 66, 464–466. doi: 10.1016/j.jinf.2012.12.003

Lau, S. K., Fan, R. Y., Luk, H. K., Zhu, L., Fung, J., Li, K. S., et al. (2018). Replication of MERS and SARS coronaviruses in bat cells offers insights to their ancestral origins. *Emerg. Microbes Infect.* 7, 1–11. doi: 10.1038/s41426-018-0208-9

Letko, M., Miazgowicz, K., McMinn, R., Seifert, S. N., Sola, I., Enjuanes, L., et al. (2018). Adaptive evolution of MERS-CoV to species variation in DPP4. *Cell Rep.* 24, 1730–1737. doi: 10.1016/j.celrep.2018.07.045

Li, F., Li, W., Farzan, M., and Harrison, S. C. (2005). Structure of SARS coronavirus spike receptor-binding domain complexed with receptor. *Science* 309, 1864–1868. doi: 10.1126/science.1116480

Li, W., Moore, M. J., Vasilieva, N., Sui, J., Wong, S. K., Berne, M. A., et al. (2003). Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. *Nature* 426, 450–454. doi: 10.1038/nature02145

Liu, Z., Xiao, X., Wei, X., Li, J., Yang, J., Tan, H., et al. (2020). Composition and divergence of coronavirus spike proteins and host ACE2 receptors predict potential intermediate hosts of SARS-CoV-2. *J. Med. Virol.* 92, 595–601. doi: 10.1002/jmv.25726

Low, D. E. (2004). Why SARS will not return: a polemic. *CMAJ* 170, 68–69. Available online at: https://www.cmaj.ca/content/cmaj/170/1/68.full.pdf

Lu, G., Hu, Y., Wang, Q., Qi, J., Gao, F., Li, Y., et al. (2013). Molecular basis of binding between novel human coronavirus MERS-CoV and its receptor CD26. *Nature* 500, 227–231. doi: 10.1038/nature12328

McKimm-Breschkin, J. L., Jiang, S., Hui, D. S., Beigel, J. H., Govorkova, E. A., and Lee, N. (2018). Prevention and treatment of respiratory viral infections: presentations on antivirals, traditional therapies and host-directed interventions at the 5th ISIRV Antiviral Group conference. *Antiviral Res.* 149, 118–142. doi: 10.1016/j.antiviral.2017.11.013

Millet, J. K., and Whittaker, G. R. (2015). Host cell proteases: Critical determinants of coronavirus tropism and pathogenesis. *Virus Res.* 202, 120–134. doi: 10.1016/j.virusres.2014.11.021

Millet, J. K., and Whittaker, G. R. (2018). Physiological and molecular triggers for SARS-CoV membrane fusion and entry into host cells. *Virology* 517, 3–8. doi: 10.1016/j.virol.2017.12.015

Modjarrad, K. (2016). MERS-CoV vaccine candidates in development: the current landscape. *Vaccine* 34, 2982–2987. doi: 10.1016/j.vaccine.2016.03.104

Paraskevis, D., Kostaki, E. G., Magiorkinis, G., Panayiotakopoulos, G., Sourvinos, G., and Tsiodras, S. (2020). Full-genome evolutionary analysis of the novel corona virus (2019-nCoV) rejects the hypothesis of emergence as a result of a recent recombination event. *Infect. Gene. Evolut.* 79:104212. doi: 10.1016/j.meegid.2020.104212

Shao, S., Xu, Q., Yu, X., Pan, R., and Chen, Y. (2020). Dipeptidyl peptidase 4 inhibitors and their potential immune modulatory functions. *Pharmacol. Ther.* 209:107503. doi: 10.1016/j.pharmthera.2020.107503

Snijder, E. J., Bredenbeek, P. J., Dobbe, J. C., Thiel, V., Ziebuhr, J., Poon, L. L., et al. (2003). Unique and conserved features of genome and proteome of SARS-coronavirus, an early split-off from the coronavirus group 2 lineage. *J. Mol. Biol.* 331, 991–1004. doi: 10.1016/S0022-2836(03)00865-9

Song, Z., Xu, Y., Bao, L., Zhang, L., Yu, P., Qu, Y., et al. (2019). From SARS to MERS, thrusting coronaviruses into the spotlight. *Viruses* 11:59. doi: 10.3390/v11010059

Takagaki, Y., Koya, D., and Kanasaki, K. (2017). Dipeptidyl peptidase-4 inhibition and renoprotection: the role of antifibrotic effects. *Curr. Opin. Nephrol. Hypertens.* 26, 56–66. doi: 10.1097/MNH.0000000000000291

Tang, X., Wu, C., Li, X., Song, Y., Yao, X., Wu, X., et al. (2020). On the origin and continuing evolution of SARS-CoV-2. *Natl. Sci. Rev.* doi: 10.1093/nsr/nwaa036. [Epub ahead of print].

Thiel, V., Ivanov, K. A., Putics, A., Hertzig, T., Schelle, B., Bayer, S., et al. (2003). Mechanisms and enzymes involved in SARS coronavirus genome expression. *J. General Virol.* 84, 2305–2315. doi: 10.1099/vir.0.19424-0

van Boheemen, S., de Graaf, M., Lauber, C., Bestebroer, T. M., Raj, V. S., Zaki, A. M., et al. (2012). Genomic characterization of a newly discovered coronavirus associated with acute respiratory distress syndrome in humans. *MBio* 3, e00473–e00412. doi: 10.1128/mBio.00473-12

Walls, A. C., Tortorici, M. A., Frenz, B., Snijder, J., Li, W., Rey, F. A., et al. (2016). Glycan shield and epitope masking of a coronavirus spike protein observed by cryo-electron microscopy. *Nat. Struct. Mol. Biol.* 23:899. doi: 10.1038/nsmb.3293

Wan, Y., Shang, J., Graham, R., Baric, R. S., and Li, F. (2020). Receptor recognition by the novel coronavirus from Wuhan: an analysis based on decade-long structural studies of SARS coronavirus. *J. Virol.* 94, e00127-20. doi: 10.1128/JVI.00127-20

Wang, N., Shi, X., Jiang, L., Zhang, S., Wang, D., Tong, P., et al. (2013). Structure of MERS-CoV spike receptor-binding domain complexed with human receptor DPP4. *Cell Res.* 23:986. doi: 10.1038/cr.2013.92

Wang, W., Tang, J., and Wei, F. (2020). Updated understanding of the outbreak of 2019 novel coronavirus (2019-nCoV) in Wuhan, China. *J. Med. Virol.* 92, 441–447. doi: 10.1002/jmv.25689

Wirblich, C., Coleman, C. M., Kurup, D., Abraham, T. S., Bernbaum, J. G., Jahrling, P. B., et al. (2017). One-health: a safe, efficient, dual-use vaccine for humans and animals against Middle East respiratory syndrome coronavirus and rabies virus. *J. Virol.* 91, e02040–e02016. doi: 10.1128/JVI.02040-16

Woo, P. C., Lau, S. K., Li, K. S., Tsang, A. K., and Yuen, K.-Y. (2012). Genetic relatedness of the novel human group C betacoronavirus to Tylonycteris bat coronavirus HKU4 and Pipistrellus bat coronavirus HKU5. *Emerg. Microbes Infect.* 1, 1–5. doi: 10.1038/emi.2012.45

World Health Organization (2003). Severe acute respiratory syndrome (SARS). *Wkly. Epidemiol. Rec.* 78:89.

Wu, A., Peng, Y., Huang, B., Ding, X., Wang, X., Niu, P., et al. (2020). Genome composition and divergence of the novel coronavirus (2019-nCoV) originating in China. *Cell Host Microbe*. 27, 325–328. doi: 10.1016/j.chom.2020.02.001

Wu, F., Zhao, S., Yu, B., Chen, Y.-M., Wang, W., Song, Z.-G., et al. (2020). A new coronavirus associated with human respiratory disease in China. *Nature* 579, 265–269. doi: 10.1038/s41586-020-2008-3

Xiao, X., Chakraborti, S., Dimitrov, A. S., Gramatikoff, K., and Dimitrov, D. S. (2003). The SARS-CoV S glycoprotein: expression and functional characterization. *Biochem. Biophys. Res. Commun.* 312, 1159–1164. doi: 10.1016/j.bbrc.2003.11.054

Zaki, A. M., Van Boheemen, S., Bestebroer, T. M., Osterhaus, A. D., and Fouchier, R. A. (2012). Isolation of a novel coronavirus from a man with pneumonia in Saudi Arabia. *N. Engl. J. Med.* 367, 1814–1820. doi: 10.1056/NEJMoa1211721

Zhang, L., Shen, F.-M., Chen, F., and Lin, Z. (2020). Origin and evolution of the 2019 novel coronavirus. *Clin. Infect. Dis*. 71, 882-883. doi: 10.1093/cid/ciaa112

Zhou, P., Yang, X.-L., Wang, X.-G., Hu, B., Zhang, L., Zhang, W., et al. (2020). A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature* 579, 270–273. doi: 10.1038/s41586-020-2012-7

Zumla, A., Hui, D. S., and Perlman, S. (2015). Middle East respiratory syndrome. *Lancet* 386, 995–1007. doi: 10.1016/S0140-6736(15)60454-8

Conflict of Interest: MR was employed by the company OGDCL, Pakistan.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Public Health

REVIEW
published: 27 August 2020
doi: 10.3389/fpubh.2020.00476

Importance of Dietary Changes During the Coronavirus Pandemic: How to Upgrade Your Immune Response

Ali Chaari, Ghizlane Bendriss, Dalia Zakaria and Clare McVeigh*

Premedical Department, Weill Cornell Medicine, Qatar Foundation, Education City, Doha, Qatar

The new coronavirus pandemic continues to spread causing further public health, social, and economic issues. The disparities in the rates of death between countries poses questions about the importance of lifestyle habits and the immune status of populations. An exploration of dietary habits and COVID-19-related death might unravel associations between these two variables. Indeed, while both nutritional excess and deficiency are associated with immunodeficiency, adequate nutrition leading to an optimally functioning immune system may be associated with better outcomes with regards to preventing infection and complications of COVID-19, as well as developing a better immune response to other pathogenic viruses and microorganisms. This article outlines the key functions of the immune system and how macronutrients, micronutrients, and metabolites from the gut microbiome can be essential in the development of an efficient immune system. In addition, the effects of intermittent fasting on the inflammatory state as well as metabolic parameters will be discussed.

Keywords: COVID-19, coronavirus, immune system, balanced diet, micronutrients, macronutrients, probiotics, intermittent fasting

OPEN ACCESS

Edited by:
Zisis Kozlakidis, International Agency for Research on Cancer (IARC), France

Reviewed by:
Annalisa Terranegra, Sidra Medicine, Qatar
Mouaadh Abdelkarim, Aix Marseille Université, France

****Correspondence:***
Ali Chaari
alc2033@qatar-med.cornell.edu

Specialty section:
This article was submitted to Infectious Diseases, Surveillance, Prevention and Treatment, a section of the journal Frontiers in Public Health

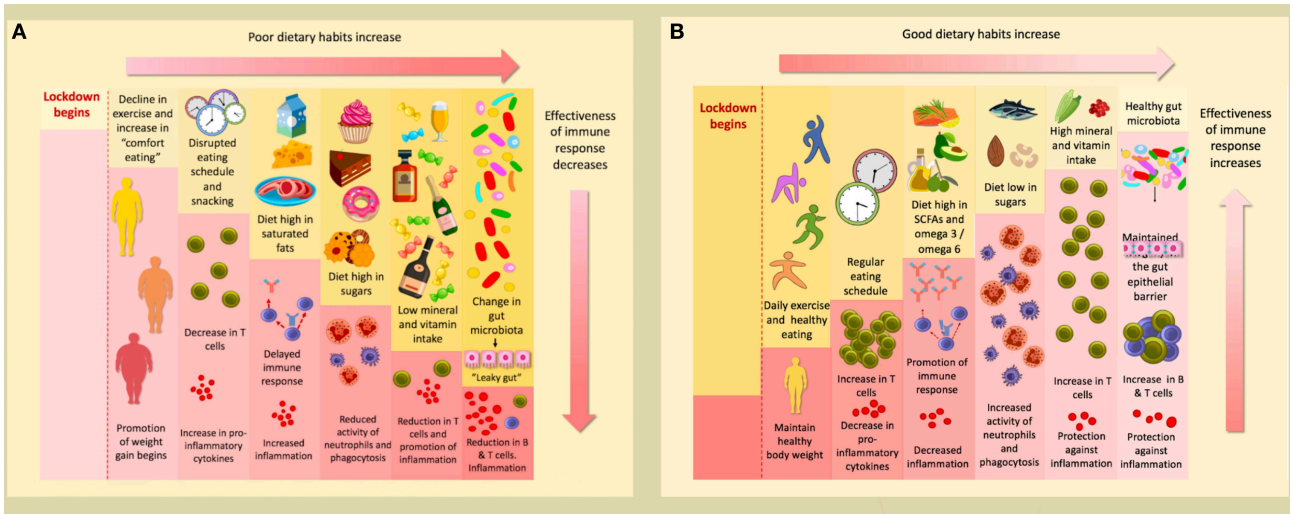
Received: *12 May 2020*
Accepted: *27 July 2020*
Published: *27 August 2020*

Citation:
Chaari A, Bendriss G, Zakaria D and McVeigh C (2020) Importance of Dietary Changes During the Coronavirus Pandemic: How to Upgrade Your Immune Response. Front. Public Health 8:476. doi: 10.3389/fpubh.2020.00476

INTRODUCTION

In the past two decades, the world has seen the emergence of three novel coronaviruses (CoV) leading to disease outbreaks that have caused considerable global health consternation: the severe acute respiratory syndrome coronavirus (SARS-CoV), the Middle East respiratory syndrome coronavirus (MERS-CoV), and the recently emerged coronavirus SARS-CoV-2 (1–3).

COVID-19 is the name of a newly identified disease caused by SARS-CoV-2, and it was originally observed as a cluster of atypical pneumonia cases occurring in Wuhan, China, in December 2019 (2). While this newly identified virus belongs to the same β-coronavirus genus as SARS-CoV and MERS-CoV, the novel disease seems to be characterized not only by mild upper respiratory infections, similar to other corona-viruses, but also by the presence of symptoms of the lower respiratory tract that are sometimes very severe (4). These mild and even asymptomatic cases have contributed to the silent spread of infections worldwide, increasing the probability of infecting high risk groups of individuals comprising immunocompromised patients and those with chronic diseases (1, 4–12). Indeed, the WHO has estimated the reproductive number (R_0) of the novel infection by SARS-CoV-2 to range between 2 and 2.5, which is higher than SARS (1.7–1.9) and MERS (<1), suggesting from the outset that COVID-19 has a higher pandemic potential (9, 10).



GRAPHICAL ABSTRACT | (A) The conditions of lockdown tend to promote poor dietary habits; a decline in exercise and increase in comfort eating promotes the weight gain that many have experienced during this time. A disrupted eating schedule and frequent snacking can result in a decrease in T cells and an increase in pro-inflammatory cytokines. A delayed immune response and increased inflammation can occur as a consequence of a diet high in saturated fats. Furthermore, a high sugar diet reduces the activity of neutrophils ad phagocytes. This will be accompanied by a reduction in T cells and promotion of inflammation if levels of minerals and vitamins are insufficient. Finally, poor dietary habits change the gut microbiota, causing "leaky gut," which results in a reduction of B & T cells, and subsequent inflammation. **(B)** Conversely, following good dietary habits and undertaking daily exercise during lockdown helps maintain a healthy weight. If a regular eating schedule is followed, with well-spaced meals, levels of T cells will actually increase, and there will be a reduction in pro-inflammatory cytokines. A diet rich in SCFAs and with a high omega 3/omega 6 ratio will promote a strong immune response and decrease inflammation. If sugars are consumed only at low levels, the activity of neutrophils and phagocytes will increase. An accompanying increase in T cells will occur if an adequate level of minerals and vitamins are consumed; they will also protect against inflammation. These affects will also be promoted if a healthy gut microbiome is maintained to preserve the integrity of the gut epithelial barrier.

Some studies have shown that patients with COVID-19 experience a dysregulation of their immune response (13). Conversely, other studies have emphasized how some individuals can recover from COVID-19 symptoms within days; an effective immune response was found to be associated with successful clinical recovery (14). Many studies have highlighted the important role of the human innate and adaptive system in COVID-19 pathophysiology (15, 16). Furthermore, there is evidence that environmental factors, such as unbalanced nutrition, toxins, and inflammation, and the sudden lifestyle changes that occur during quarantine/lockdown can cause physicochemical and psychological stress. These factors may lead to a compromised immune system and deregulate the immune system, making the human body more vulnerable to viral infections (17–20).

An optimal nutritional state has been found to be essential for a well-functioning immune system and for the protection against viral infections (21). Furthermore, malnutrition and/or an unbalanced diet represent an important cause of immunodeficiency worldwide, with infants, children, adolescents, and the elderly being the most affected (22, 23). In this context, deficiencies in essential nutrients are associated with an impairment of cell-mediated immunity, phagocyte function, complement system, and cytokine production in humans (22, 23). Moreover, deficiency in micronutrients such as vitamins, minerals, and polyphenols has been shown to have profound consequences for immune system functioning and susceptibility to infection. Carotenoids, vitamins, selenium, zinc, and polyphenols, as well as many other nutrients, have been shown to modulate the immune system. Furthermore, dietary manipulation of these micronutrients has been shown to alter immune function (21, 24–26). Nutritional excess of carbohydrates, saturated fats, coupled with physical inactivity leading to obesity, can also deregulate the immune system of the host thereby increasing susceptibility to infection (21, 27). Unfortunately, the quarantine and self-isolation of many individuals during the current pandemic promotes these unhealthy behaviors.

This narrative review principally aims at revealing the benefits of balanced nutrition in prevention and treatment of viral infection, by strengthening the immune system. We will be discussing benefits of a number of macro and micronutrients as well as their mechanisms of action.

In addition, evidence is emerging that chronic diseases are strongly associated with the severity of the symptoms and prognosis (11) but the mechanisms explaining this relationship are still unclear and are being explored. Unfortunately, only a limited amount of clinical data is available to draw direct conclusions on the potential of nutritional changes in protecting individuals from COVID19. However, we believe that it is important to note that any changes in lifestyle can also greatly impact chronic diseases in non-infected chronic patients with a high risk for severe COVID-19 disease and thereby *indirectly* affect their response to SARS-CoV-2 infections. Therefore, information regarding the effects of nutritional changes, including fasting, in reversing dysbiosis and chronic

diseases in non-infected High Risk for Severe Illness (HRSI) individuals is also included in this paper.

HUMAN CORONAVIRUS INFECTION AND THE HOST'S IMMUNE SYSTEM

Components of the Human Immune System and Lines of Defense Against Viral Infection

The first line of immune defenses includes the physical and chemical barriers that attempt to block the entry of microbes. When these barriers are breached, the microbes will be fought by the components of the internal innate immune system which is composed of leukocytes and defensive proteins that act immediately and non-specifically to eradicate infections (28). If innate immunity fails to eliminate the infection, the adaptive immune system will be activated. T and B lymphocytes are the adaptive immune cells which are able to recognize antigens with high specificity (28, 29). **Table 1** summarizes the major functions of the innate and adaptive immune cells.

The immune response is triggered by the interaction between the pattern recognition receptors (PRRs) of the host cells and the pathogen associated molecular patterns (PAMPs) (41). The antiviral defense is initiated when PRRs such as Toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) or NOD-like receptors (NLRs) bind to viral PAMPs such as DNA, RNA, or proteins (42). This interaction induces some signaling cascades through the activation of different families of transcription factors (43, 44). Type I and Type II interferons (IFN-I and IFN-II) are cytokines produced in response to viral infections (45). IFN-I (IFN-α and β) are produced by various types of cells and interfere with viral replication which creates an antiviral state through various mechanisms (46–48). In addition to directly inhibiting viral replication, IFN-I can modulate the innate and adaptive immunity including the activation of the cytotoxic activity of natural killer (NK) cells and cytotoxic CD8+ T lymphocytes (CD8+ CTL) cells which are essential to eradicate the virally infected host cells. Furthermore, IFN-I can stimulate the production of IFN-γ (IFN-II) by NK cells (49). IFN-γ promotes the macrophages classical pathway (M1) which induces inflammation and promotes the intracellular killing mechanisms. Furthermore, IFN-γ stimulates the differentiation of CD4+ T helper (Th) lymphocytes into Th1 which themselves are major producers of IFN-γ (35). Conversely, Th2 activate the alternative pathway of macrophages (M2) which suppresses inflammation and promotes the repair mechanisms (36). Therefore, the Th1 response, together with the cytotoxic activities of NK and CD8+ CTL, are vital antiviral mechanisms (28, 50).

TABLE 1 | Summary of the major functions of the innate and adaptive immune cells.

Innate leukocytes	**Description and function**	**References**
Mast cells	Produce/secrete proinflammatory mediators such as cytokines, eicosanoids, and vasoactive amines such as histamine, which causes vasodilation and increases vascular permeability.	(30)
Macrophages	Phagocytes that ingest and destroy microbes. They also produce inflammatory cytokines.	(31)
Monocytes	Circulating phagocytes which can ingest microbes in blood. They migrate to tissues under inflammatory conditions and differentiate to macrophages. They also produce inflammatory cytokines.	(31)
Neutrophils	Circulating phagocytes/granulocytes. They migrate to tissues under inflammatory conditions and destroy microbes by phagocytosis and degranulation. They also produce inflammatory mediators.	(31)
Eosinophils	Circulating granulocytes. They migrate to tissues under inflammatory conditions and kill parasites.	(32)
Basophils	Circulating granulocytes. They migrate to tissues under inflammatory conditions and kill parasites.	(32)
Natural Killer (NK) cells	They are responsible for killing host cells that are infected, stressed, or damaged. Therefore, they play an important role in the eradication of intracellular pathogens and tumor cells. They also produce inflammatory cytokines.	(33, 34)
Dendritic cells (DC)	They function as antigen presenting cells (APC) which mediate the transition from innate to adaptive immunity. If the innate immune system fails to eliminate infection, DC capture and process protein antigens and present them to T lymphocytes. They produce inflammatory cytokines.	(28, 29)
Adaptive leukocytes (lymphocytes)	**Function**	**References**
CD4+ T cells	Upon activation by APC, they become helper T cells (Th1, Th2, or Th17). Some CD4+ T cells are regulatory (Treg).	(28, 29)
	Th1: Activate the M1 pathway of macrophages which induce inflammation. They also produce inflammatory cytokines.	(35)
	Th2: Activate the M2 pathway of macrophages which suppress inflammation.	(36)
	Th17: Produce IL-17 which activates and recruits inflammatory leukocytes to various tissues.	(37)
	Treg: Regulatory CD4+ T cells which have immunosuppressive effect.	(38)
CD8+ T cells	Upon activation by APC, they become cytotoxic T cells (CTL) which are responsible for killing infected, stressed, or damaged host cells.	(28, 29)
B cells	When activated, they produce antibodies that neutralize pathogens and enhance the effector mechanisms of other immune cells such as phagocytes.	(39, 40)

The inflammasome is an important structure in the antiviral defense which is assembled when cytosolic viral molecules bind to NLR. It induces the activation and secretion of interleukin (IL) 1β which is a potent pro-inflammatory cytokine. Moreover, it induces pyroptosis leading to the host cell death and consequently the control of viral infection (51). Tumor necrosis factor-α (TNF-α) is another potent pro-inflammatory cytokine that can cause host cell apoptosis (52). Both TNF-α and IL-1β induce the expression of adhesion molecules by endothelial cells which is essential for the migration of leukocytes across capillaries as part of the inflammation cascade (52, 53). Inflammation could also be induced by a wide range of cytokines such as IL-6, which, in addition to its pro-inflammatory function, together with transforming growth factor (TGF)-β, stimulate the differentiation of "CD4+ Th cells or Th cells" into the proinflammatory Th17 subset (54, 55). Th17 cells are characterized by the production of IL-17 which plays an essential role in the antiviral defense by activating and recruiting inflammatory leukocytes in various tissues (37). Furthermore, IL-17 was reported to promote an effective Th1 and CD8+ CTL responses in addition to the enhancement of humoral immunity by promoting B cell proliferation and differentiation into plasma cells during viral infections (37, 56). Humoral immunity is an essential arm of the antiviral defenses, providing the antibodies that neutralize the virus and enhancing the effector mechanisms of other immune cells such as phagocytes (39, 40). IL-17 could be also produced by a wide range of immune cells such as NK and $\gamma\delta$ T cells (57–59). $\gamma\delta$ T cells are a subgroup of T cells that have a different structure of T cell receptors compared with conventional T cells ($\alpha\beta$ T cells) which can bind to non-peptide antigens. It has been shown that $\gamma\delta$ T cells link innate and adaptive immunity and work as antigen presenting cells (APC) to activate CD4+ Th and CD8+ CTL in addition to their capacity to produce cytokines and lytic enzymes which take part in controlling viral infections (60).

Another type of pro-inflammatory cytokines is the chemokines that induce inflammation by functioning as leukocytes chemoattractants. Examples of chemokines that take part in antiviral defense are monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1 alpha (MIP-1), IFN-γ inducible protein (IP-10) and IL-8 which are summarized in **Table 2**.

Additionally, some cytokines are required for the development, proliferation, differentiation, and survival of leukocytes and may therefore act as pro- or anti-inflammatory cytokines. For example, granulocyte colony-stimulating factor (G-CSF) enhances the production and function of neutrophils and macrophages and consequently could function as a pro-inflammatory cytokine (66, 67). On the other hand, both IL-7 and IL-2 play a pivotal role in the development and homeostasis of lymphocytes and may induce inflammation (68, 69). However, IL-2 is also required for the development and function of regulatory T cells (Treg) (70). Accordingly, IL-2 may have a dual function as pro-inflammatory or anti-inflammatory cytokine (38, 69). Inflammation could be suppressed by the anti-inflammatory cytokines which are summarized in **Table 2**.

Despite the vital defensive role of inflammation as a major immune response, it is important to note that in several viral infections, the tissue damage is not directly caused by the virus, it is instead the result of an exuberant inflammatory response to the viral infection (73, 74).

Human Immune Responses to SARS-CoV-2 Infection

MERS-CoV, SARS-CoV, and SARS-CoV-2 are β-coronaviruses that can cause fatal respiratory tract infections and extra-pulmonary manifestations (75–77). SARS-CoV-2 binds to the angiotensin-converting enzyme 2 (ACE2), which it uses as a receptor to enter the cell (78, 79). ACE2 proteins, part of the renin- angiotensin system (RAS), are found at several locations, including the olfactory epithelium and the gut and are numerous throughout the respiratory epithelial tissue of the lung, kidney, intestine, and blood vessels (80). This may be the cause behind the high incidence of bronchitis and pneumonia in severe COVID-19 infected patients. It has been shown that ACE2 is responsible for the degradation of Angiotensin II resulting in the formation of Angiotensin 1-7, thereby, negatively regulating RAS (81, 82). Besides the role of ACE2 to serve as a functional receptor for SARS-CoV-2, it has been shown that ACE2 is implicated in many pathologies including diabetes, cardiovascular diseases (CVD), and lung diseases (82–84). SARS-CoV-2 appears to use different amino acids in its spike protein for binding the ACE2 receptor with more affinity than previous SARS viruses (85, 86). Interestingly, the latest studies have shown that, after infection, some cellular processes downregulate ACE2 expression (87). Destruction of ACE2 further increases the activity of angiotensin II, which has pro-inflammatory, pro-oxidative, vasoconstrictive, and pro-thrombotic effects that can lead to the thrombotic changes and organ failure that were noted in COVID19 patients and which contributed to death (88). In fact, it seems that after viral infection, ACE2 could play a key protective role in the progression of the disease and the severity of the respiratory distress syndrome (89). A study by Imai et al. (89) published in Nature have shown that ACE2 protects mice form severe acute lung injury after sepsis. Sepsis is characterized by oxidative stress, systemic inflammation, and organ failure that is due to excessive free radical production.

Based on the previous studies conducted on SARS-CoV and MERS-CoV, it could be predicted that the innate immune response against SARS-CoV-2 may start when the viral molecules are recognized by TLRs, RLR, or NLR. This interaction triggers the inflammatory response and stimulates the production of IFN-I which controls viral replication (77). However, it was also reported that SARS-CoV and MERS-CoV may evade the innate immune response by interfering with the IFN-I signaling pathways through various mechanisms. Failure to initiate or complete the IFN signaling cascades during the early phase of infection may result in an uncontrolled viral replication. This may lead to the recruitment of neutrophils and monocytes/macrophages to the infected tissues which results in the excessive production of pro-inflammatory cytokines (90). Accordingly, it could be hypothesized that the exaggerated

TABLE 2 | Summary of the major functions of cytokines and chemokines.

Cytokine	Function in antiviral immune response	Mechanism of action	References
IFN-I (IFN-α and β)	Antiviral	Interfere with viral replication, activate NK cells, and induce the production of IFN-γ.	(49)
IFN-II (IFN-γ)	Pro-inflammatory	Activates the M1 pathway and promote Th differentiation to Th1.	(35)
IL-1β	Pro-inflammatory	Induces the expression of adhesion molecules by endothelial cells and induce pyroptosis.	(51, 52)
TNF-α	Pro-inflammatory	Induces the expression of adhesion molecules by endothelial cells and induce apoptosis.	(53)
IL-6	Pro-inflammatory	Promotes Th differentiation to Th17 and induce the production of CRP which is part of the acute phase inflammatory response.	(54, 55)
IL-17	Pro-inflammatory	Recruits inflammatory leukocytes to the site of infection, promote an effective Th1 and CD8+ CTL responses and enhance humoral immunity.	(37, 56)
MCP-1	Pro-inflammatory/chemoattractant	Recruits monocytes from blood stream to the site of infection.	(61)
MIP-1α	Pro-inflammatory/chemoattractant	Recruits inflammatory leukocytes to the site of infection.	(62)
IP-10	Pro-inflammatory/chemoattractant	Recruits inflammatory leukocytes and enhance inflammation by promoting the Th1 response.	(63, 64)
IL-8	Pro-inflammatory/chemoattractant	Recruits neutrophils to the site of infection which enhances inflammation.	(65)
G-CSF	Pro-inflammatory	Enhances the production of neutrophils and macrophages and enhances phagocytosis.	(66, 67)
IL-7	Pro-inflammatory	Promotes the development, proliferation, and survival of lymphocytes and suppress the expression of inhibitory molecules by T cells.	(68)
IL-2	Pro-inflammatory/Anti-inflammatory	Enhances proliferation and survival of Th1, Th2, Th17, and Treg.	(69, 70)
IL-4	Anti-inflammatory	Activates the M2 pathway and promote Th differentiation to Th2.	(71)
IL-10	Anti-inflammatory	Regulates inflammation.	(72)

damaging inflammatory response observed in COVID-19 patients is at least partially attributed to the suppressed/delayed IFN-I pathways accomplished by SARS-CoV-2. Furthermore, in severe COVID-19 cases there is a diminished response of Th1 cells (13).

Several studies have documented that levels of cytokines and chemokines vary according to disease stage and severity of COVID-19. For example, one study showed that plasma levels of IL-2, IL-6, IL-8, IL-10, and TNF-α, were found to be higher in patients with severe infection than those with mild to moderate infection (13). Another study showed a similar trend, with plasma concentrations of IL-2, IL-7, IL-17, IL-10, MCP-1, MIP-1A, IP10, and TNF-α being observed to be higher in COVID-19 patients undergoing treatment in intensive care units than in any other category of COVID-19 patients (91).

In one report, analyzing 99 cases in Wuhan, Zhou and colleagues observed an increase in the total neutrophils (38%), an increase in serum IL-6 (52%), an increase in C-reactive protein (CRP) (84%), and a decrease in total lymphocytes (35%) (92). In another report from Wuhan, analyzing 41 patients, an increase in the total neutrophils and a decrease in the total lymphocytes has been shown, which also correlate with disease severity and death (91). Furthermore, the decreased level of lymphocytes observed by (90), could be explained by the ability of SARS-CoV-2 to infect T lymphocytes, which leads to apoptosis of lymphocytes and consecutive lymphocytopenia (4, 93). In fact, it was found that the absolute count levels of CD4+ and CD8+ T cells were significantly lower in subjects with a severe SARS-CoV-2 infection (94–96). In addition to T cells, the reduction of B cells and NK cells are seen in COVID-19 (13, 97). Therefore, the reduced adaptive immune response against the virus, manifested by an impaired T-cell function, may contribute to the uncontrolled secretion of the pro-inflammatory cytokines in what is known as a "cytokine storm" accompanied with a multi-organ failure (8, 98). Interestingly, one study illustrated how an otherwise healthy individual with a robust immune system is capable of achieving an efficient clearance of SARS-CoV-2, accompanied by clinical recovery after 13 days and full recovery at day 20 after infection (14).

The impact of comorbidity is yet another factor that may affect the outcome of COVID-19. It has been reported that factors such as obesity, diabetes and CVD may increase the risk of progression and mortality among COVID-19 patients (99). One factor that may link such diseases to the increased severity and progression of COVID-19 is inflammation. For example, obesity is associated with metabolic alterations which may dysregulate the immune response through various mechanisms (100). Furthermore, obesity was found to be associated with the increased production of IL-6, TNF-∞, MCP-1, and CRP leading to chronic and low-grade inflammation which may result in defective innate immunity and cause the development of type 2 diabetes and CVD (100, 101). Likewise, the association

between diabetes, CVD, and chronic inflammation has been well-established (102, 103). Additionally, studies have shown that ACE2 expression is significantly increased in obese individuals, as the RAS upregulates ACE2 to protect the heart. However, because of this increased ACE2 expression, obese individuals are thought to be more exposed to the SARS-CoV2 viral spread into the lungs. Treatment and close management of obesity is an important approach that needs to be considered to prevent patients from being infected and developing complications.

Therefore, it could be elucidated that the efficiency of the immune response, which is controlled by multiple factors including nutrition, may dictate the outcome of COVID-19. The following section presents a review of the nutritional components that were shown to boost the immune system, including, but not limited to viral infections and coronaviruses.

THE ROLE OF NUTRITION IN IMMUNE FUNCTION

A balanced, adequate diet is required for the cells of the immune system in order to function optimally. During situations with increased requirements (e.g., infection, stress, and pollution), the immune system is activated and thus increases the demand for energy. A balanced, optimal diet strengthens the immune response and supports the function of the immune cells not only by producing an effective response against pathogens, but also by resolving infections in a short time thus avoiding any further chronic inflammation (104). Various nutrients are involved in this process. This section highlights some that have been shown to play specific roles in the development and maintenance of an effective immune system.

Role of Macronutrients in the Immune Function

Effect of Dietary Fats in the Immune System

Dietary fats are mostly triglycerides and are among the most important sources of nutrition in humans if taken appropriately. Many food sources contain various types of fatty acids, such as olive oil which is rich in monounsaturated fatty acids, animal products rich in saturated fats (but also with large proportions of monounsaturated and polyunsaturated fatty acids depending on the origin), plants rich in alpha linolenic acid, and nuts and seeds (such as walnuts and linseed), rich in omega 3 polyunsaturated fatty (105). Fatty acids are known to play diverse roles in immune cells (106, 107). Dietary fats are important for absorption of liposoluble vitamins A, D, E, and K (which are also involved in the immune system), as well as permeability and stability of immune cell membranes (108).

Short chain fatty acids (SCFAs), like acetate, propionate, and butyrate can be provided by many fermented foods made by bacterial fermentation such as cheese, butter, pickles, soy sauce, yogurt, and alcoholic beverages (109–113). Many studies have shown that SCFAs exert anti-inflammatory properties and present immunomodulatory potential *in vitro* (114, 115). SCFAs are able to regulate the activation, recruitment, and differentiation of immune cells, including neutrophils, dendritic cells (DCs), macrophages, and T lymphocytes (116). A study by Liu and colleagues showed that SCFAs not only reduced the production of pro-inflammatory factors, including TNF-α, IL-1β, IL-6, but also enhanced the production of the anti-inflammatory cytokine IL-10 (117).

Many studies have shown that palmitoleic acid (PA) (a monosaturated fatty acid belonging to the omega-7 group), also presents anti-inflammatory properties *in vitro* (118, 119). Dietary sources of palmitoleic acid include a variety of animal oils, vegetable oils, and marine oils. A recent study evidenced the role of the palmitoleic acid in decreasing pro-inflammatory cytokine expression in cultured macrophages characterized by a decrease in Th1 and Th17 response (120). Another important constituent of dietary fats is polyunsaturated fatty acids, which can be further subdivided into omega-3 and omega-6 fatty acids. Many studies using a variety of models show that a decrease in omega-6/omega-3 ratio has anti-inflammatory effects (121–125). A study using mice reported that the omega-3-derived lipid mediator protectin D1, significantly reduced influenza virus replication (126). Moreover, a randomized controlled trial showed that omega-3 supplements were able to lower inflammation in healthy middle-aged and older adults (124). The data showed that administration of 1,25 and 2.5 g/d of omega-3 decreased the IL-6 serum level by 10 and 12%, respectively (124). Another randomized control study showed that supplementation of omega 3 for 12 weeks reduced the production of IL-6, and lowered anxiety by 20%. These changes were accompanied by a decreasing ratio of omaga-6/omega-3 and consequent reductions in IL-6 and TNF-α production (127). Although the beneficial effect of omega-3 has been revealed by many studies, a caution with dose and the status of the body should be taken into consideration when this compound is taken. On the other hand, it has been shown that saturated and polyunsaturated omega-6 fatty acids present pro-inflammatory properties (107, 128). Furthermore, omega-6 fatty acids are precursors of potent lipid mediator signaling molecules, termed "eicosanoids," which have important roles in the regulation of inflammation, and the eicosanoids derived from omega-6 also present pro-inflammatory properties (129). However, it should be mentioned that not all omega-6 have pro-inflammatory characteristics. Gamma-linolenic acid (GLA, 18:3n-6) is a precursor of eicosanoids, which is found in human milk and several botanical seed oils but is typically consumed as part of a dietary supplement. Several studies have shown that GLA can attenuate inflammatory responses (130, 131). Furthermore, it has been shown that polyunsaturated fatty acids are able to activate the peroxisome proliferator-activated receptors γ (PPAR-γ), thus decreasing the pro-inflammatory cytokines (132). For example, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) interact with PPAR-γ and leads to the inhibition of nuclear factor- κB (NF-κB), a key transcription factor of pro-inflammatory cytokine production (133). On the other hand, saturated fatty acids have been shown to trigger the secretion of pro-inflammatory mediators from various cell types, including macrophages (134, 135), adipocytes (136), astrocytes (137), and endothelial cells (138). An *in vitro* study also showed that the addition of palmitic acid to infected cells, by different

strains of the influenza A virus, increased the cellular lipid content and thus increased the replication of the virus (139). This effect of palmitic acid has not been replicated for coronaviruses.

It has been reported that high-fat diets lead to increasing circulating pro-inflammatory cytokine and neutrophil levels, resulting in a poorer response to pandemic H1N1 influenza A virus (pH1N1) vaccination (140). In the same context, Milner and colleagues state that "Obesity has been identified as an independent risk factor for severe or fatal infection with 2009 pandemic H1N1 influenza (2009 pH1N1), but was not previously recognized for previous pandemic or seasonal influenza infections" (141). In this study, the authors showed that obese mice had elevated viral titers, greater lung inflammation, as well as increased inflammatory cytokine levels and damage, and more memory CD8+ CTL in the lung airways (141, 142). Moreover, HFD leading to obesity (animal model of obesity) can exacerbate inflammation or infection in the host, and consequently increase the mortality. This has been shown in obese mice infected with the influenza virus (143, 144), which was attributed to a delayed antibody response (141). In fact, infection of obese mice with the 2009 pandemic H1N1 influenza virus resulted in an elevation of pro- inflammatory cytokine concentrations in circulation, but a lower response of IFN-β and pro-inflammatory cytokine concentrations in the lungs, compared to lean mice (144). Similarly, another study with obese mice infected with the influenza virus showed that IFN-α and β were minimally expressed and there was a notable delay in expression of the pro-inflammatory cytokines IL-6 and TNF-α (143). The lower level of IFN-α and β leads to a less effective immune responses against viral agents (145). In this context, it has been shown that there is strong association between severity of COVID-19 disease and obesity (146). Thus, during the lockdown, individuals with a tendency for obesity and other metabolic disorders should avoid or reduce high fat meals since it has been shown that high fat diet have a detrimental role, downregulating ACE2 (147). Deregulation of ACE2 receptors in the airways allows easier entrance of the virus and leads to the increased angiotensin II release. In turn, this can cause vascular (endothelial) trauma and micro-thrombo-embolism in various organs, leading to multiple organ failure (82, 88).

Furthermore, high-fat dietary intake has been proven to be responsible for the alteration of microbial composition in the intestine by increasing the ratio of Firmicutes to Bacteroidetes leading to an increase in intestinal permeability. This may cause systemic inflammation thus affecting the immune system (140, 148). Trottier and colleagues observed induced inflammation in the immune system in mice that had been fed a high-fat diet. This was accompanied by a modest change in bone marrow composition and a slight increase in the percentage of lymphocytes (149).

In summary, the *in vitro* and *in vivo* studies using animal models indicate that fatty acids can directly modulate either negatively (high-fat diet, saturated and polyunsaturated omega-6 fatty acids) or positively (polyunsaturated omega-3, monounsaturated, and short-chain fatty acids) thereby affecting the immune response and influencing infection susceptibility (140) (**Figure 1**). However, a recent study in mice has shown that short term feeding (3–6 weeks) either with low-fat or high fat diets, rich with omega-3, omega-6 or monosaturated fatty acids, did not significantly influence the susceptibility of mice to viral infection, morbidity, viral titers in the lungs and liver, recovery time, or mortality (125).

Effect of Dietary Carbohydrates in the Immune System

Carbohydrates are nutrients found mainly in vegetables, fruits, and cereals and can be divided into simple sugars and oligo- or poly-saccharides. The recommended daily dietary allowance of carbohydrates is 130 g/day (150). Carbohydrates consumed as part of balanced diet are healthy but can be toxic if overconsumed. Carbohydrates are the most important fuel source and are necessary for the normal functioning of immune cells. Although an increase on lymphocytes during anaerobic glycolysis has been shown—which is an indicator of the increase of glucose as a fuel—during lymphocyte proliferation the use of this micronutrient as a source of energy decreases (151). Moreover, carbohydrates have an important impact on the immune system because of their ability to prevent the decrease of the number of cells conjoint to apoptosis (108). This fact is very important for COVID-19, because in severe cases there is an increase in apoptosis of lymphocytes.

On the other hand, a recent study showed that during times of stress (comparable to what many are facing during the COVID-19 pandemic) many people change their dietary behavior and tend to be drawn to unhealthy, high-sugar foods (152). A diet based on overconsumption of simple carbohydrates can lead to metabolic syndrome, an increase in abdominal fat, hyperglycemia, and type 2 diabetes, as well as dysregulation in the immune responses (151, 153). A recent paper by Goldberg and colleagues reported that feeding mice an energy dense, high-fat, low-carbohydrate ketogenic (keto) diet conferred protection in the context of a potentially lethal influenza infection. The authors identified that an energy dense, high-fat, low-carbohydrate ketogenic (keto) diet promoted the expansion of γδ T cells in the lung, leading to a conclusion that a keto diet may present a viable avenue toward preventing or alleviating influenza disease (154). Although this outcome was specific to mice and not to humans, it cannot be ignored that a keto diet may have beneficial effects for people with type 2 diabetes and other metabolic disorders (155–157) who have higher risk of complications if infected with SARS-CoV-2 (158). In this context, it has been revealed for example that 5.3–20% of COVID-19 patients in Wuhan had compromised innate immune responses because of diabetes (159, 160). A low carbohydrate diet has positive effects in people with type 2 diabetes (161) which may alleviate the severity of infection by SARS-CoV-2. Additionally, severe COVID-19 cases have exhibited increased catabolism, and therefore have increased energy requirements (162).

Effect of Dietary Proteins and Amino Acids on the Immune System

Proteins are considered the building blocks of life and their monomeric component, the amino acids, are considered key regulators of various pathological and physiological processes,

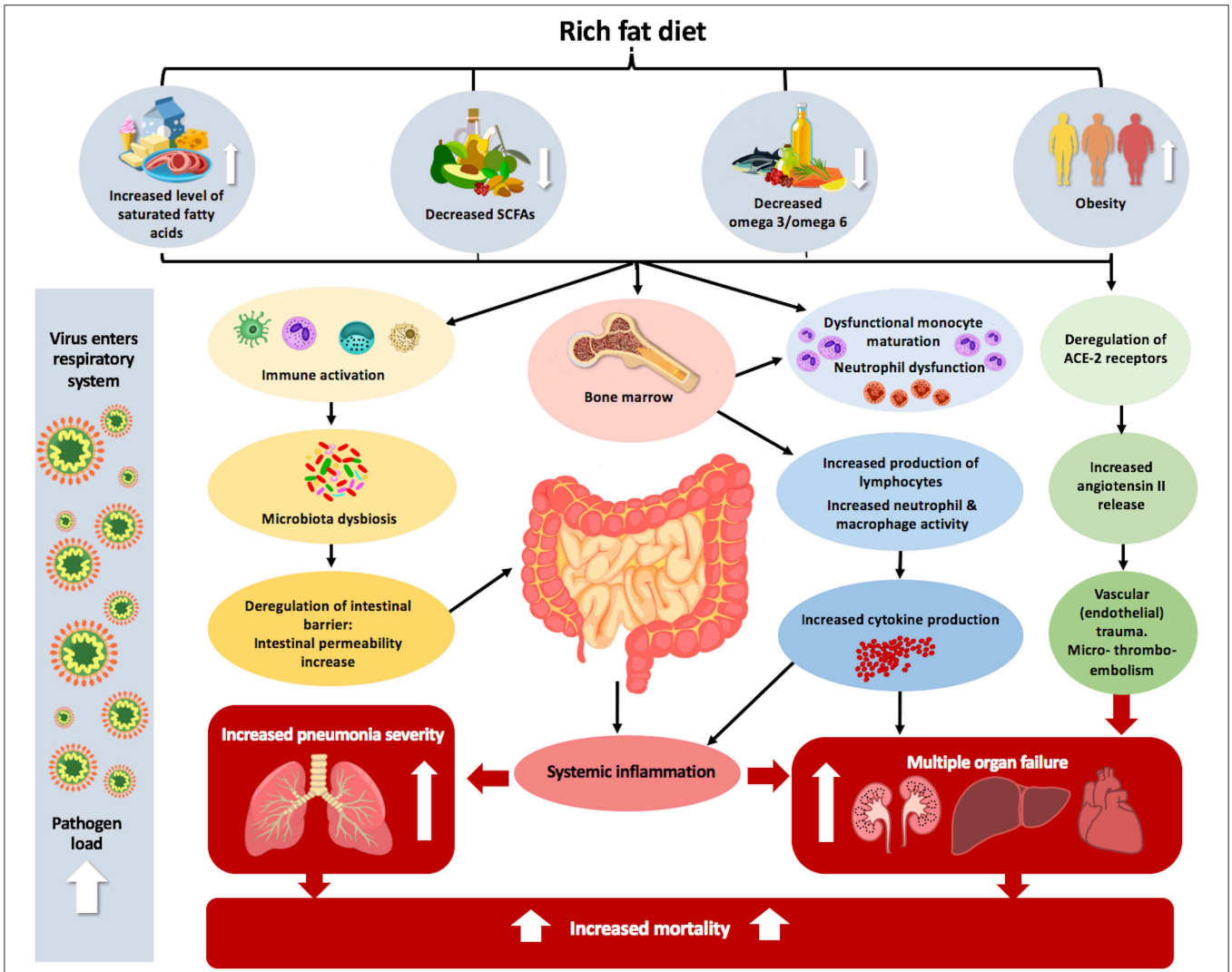


FIGURE 1 | The effect of inadequate fat intake and obesity on the pathophysiology of COVID-19. Increased dietary level consumption of saturated fatty acids, decreased level of SCFAs and omega 3/omega 6 combined with obesity can lead to immune activation. This immune activation can alter microbial composition in the intestine, leading to dysbiosis, which consequently enhances systemic inflammation. The complexity of the intestinal microbiota is correlated with dysfunctional monocyte maturation and neutrophil dysfunction in the bone marrow. Obesity also leads to deregulation of ACE2 receptors in the lungs, which predisposes and makes entrance the virus easier and leads to increased angiotensin II release, which causes vascular (endothelial) trauma and micro-thrombo-embolism in various organs, leading to multiple organ failure. Altogether, these different factors that lead to the body being unbalanced can increase pneumonia severity and mortality, which is more acute in the case of lung viral infection.

including immune responses (163). The recommended daily dietary allowance of proteins is 19–56 g/day (150). It has been demonstrated that a deficiency of dietary protein and accompanying reduced concentrations of most amino acids in plasma, impairs the immune function and increases the susceptibility of humans to infectious diseases (164). A deficiency in protein intake is associated with the alteration of one of the first lines of defense against pathogens: the physical barrier. This deficiency is accompanied by thinner collagen and connective tissue, reducing the number of antibodies in the physical barrier, which results in a favorable environment for the aggressor (165). Moreover, the protein-energy malnutrition associated with chronic diseases has been recognized as a virulence factor for severe COVID-19 because it can deregulate immune cell activation leading to increasing inflammation in the lungs and longer viral persistence (133, 166). Moreover, it has been shown that COVID-19 patients require a diet rich in high energy nutrients (105–160 kj/kg/day or 25–40 kcal/kg/day) and proteins (167–170). In this context a protein intake >1 g/kg/day (up to 1.5–2 g/kg/day) has been proposed in COVID-19 patients that do not show any chronic renal insufficiency (22, 167).

There is increasing evidence on the important role of amino acids in the enhancement of the immune response, as well as in the reduction of an over-reaction, such as inflammation

and autoimmunity (163). Thus, amino acids can regulate the activation of T and B lymphocytes, macrophages, NK cells, and the production of antibodies and cytokines (164, 171–173). Many amino acids like glutamine, arginine, tryptophan, cystine/cysteine, glutamate, histidine, and branched-chain amino acids are important for immune function (163). Some of them are discussed below.

Glutamine

This amino acid is the most abundant and versatile amino acid in the body. Research has shown that in health and disease, the rate of glutamine consumption by immune cells is similar to or greater than glucose consumption (174, 175). In fact, a decreasing level of glutamine in the plasma leads to: (1) a reduction in human B cell differentiation as well as a decrease in antibody production (176); (2) a suppression of T cell proliferation and decrease in IL-2; and (3) downregulation of major histocompatibility complex (MHC) class II antigen expression on human macrophages and inefficient phagocytosis (177).

Arginine

For many years, a diet rich in arginine, which is found abundantly in meats and nuts, often combined with other micro- and macronutrients, has been used as a mechanism to boost the immune system (178). It was reported that in experimental animals housed under stressful conditions, arginine supplementation was able to restore the reduced number of T cells to normal (163). Another study showed that L-arginine consumed through the diet can boost the activity of T cells. In fact, this study showed that an increase in the level of L-arginine reorganized the metabolism of the T cells, which made them more effective in fighting tumors and gave them a longer lifespan (179).

The Role of Micronutrients in the Immune Function

Vitamins and other micronutrients are essential constituents of the human diet that have long been known to influence the immune system (165, 180). A deficiency in these micronutrients affects the innate and adaptive immune system response, leading to dysregulation of the balanced host response (181). Many studies have shown that vitamins A, B, C, D, E, minerals zinc, iron, magnesium, selenium, iodine, copper, and polyphenols among other micronutrients, have an important effect in supporting the immune system (182).

Vitamins

Vitamin C

Vitamin C is an essential micronutrient for humans that contributes to enhancing the immune response by supporting the innate and the adaptive immune system. The recommended daily dietary allowance of this micronutrient is 25–90 μg/day (150), and a deficiency in vitamin C deregulates the barrier function against pathogens, increases oxidative damage, and decreases phagocytosis (183, 184). In other words, vitamin C deficiency results in impaired immunity and increases the incidence and severity of pneumonia and other infections (182). Various studies showed that supplementation with a high dose of vitamin C stimulates phagocytic and T-lymphocytic activity in response to infection by increasing cytokine production and synthesis of immunoglobulins (182) and can help severely ill patients in intensive care to recover more quickly (182). A randomized, double-blind placebo-controlled trial in the UK showed that the administration of 200 mg/day of vitamin C to elderly patients with pneumonia reduced respiratory symptoms, mainly in patients with more acute respiratory infection (185). In a recent meta-analysis of nine randomized controlled trials, it has been shown that administration of a high dose of vitamin C (700–800 mg/day) against common cold virus infections lead to a reduction of the duration of infection and a shorter time of confinement (186). Although the used doses to treat pneumonia are higher than the RDA, a recent NIH document revealed that a diet with 1.5 g/kg body weight of vitamin C is safe and has no major adverse events (187). In fact the use of such high doses to treat infection, rather than the normal recommended doses, could be explained by the fact that during infection, the level of vitamin C decreases and the requirements of an infected person increases with the severity of the infection (188).

Vitamin D

Vitamin D is a fat-soluble vitamin that is naturally present in very few foods, but is available as a dietary supplement, and is produced by our body in response to sun exposure. The RDA of this micronutrient is 15–20 μg/day (150). Vitamin D has the capacity to maintain the structural and functional integrity of mucosal cells in innate barriers, such as the skin and the respiratory tract, which is very important during viral infection. In fact, this vitamin increases the tight junction protein expression, E-cadherin, and connection 43 in the gut, supporting the gut barrier (182). Moreover, vitamin D has various functional roles: it increases the differentiation of monocytes to macrophages (189) and it promotes the movement and phagocytic ability of macrophages (182). Also, this vitamin increases superoxide synthesis (182), reduces the expression of pro-inflammatory cytokines, and increases the expression of anti-inflammatory cytokines by macrophages (190, 191), all of which may enhance immune system reactivity. Vitamin D presents stimulatory effects in the innate immune system, promotes the production of Treg (182), and promotes antigen processing. A study conducted by Cannell and colleagues showed that calcitriol, an active form of vitamin D, was able to reduce the incidence of respiratory infections in children during epidemic influenza by restoring the immune function of macrophages (192). A recent review recommended that people at risk of influenza and/or COVID-19 take 250 μg/day of vitamin D3 for a few weeks followed by 125 μg/day (193). The same review stated that in order to treat infected people with COVID-19, higher vitamin D3 doses might be useful (193). A recent study with a group of 780 COVID-19 patients revealed that most positive patients with insufficient or deficient vitamin D status died (194). Moreover, Rhodes and colleagues highlighted that there is a low population mortality from COVID-19 in countries south of latitude 35 degrees North, supporting the hypothesis that vitamin D is a cofactor determining the severity of the infection and

then the immune system response (195). Besides the various roles that vitamin D presents, this micronutrient could play a direct role in virus-receptor binding. In fact, it has been shown that vitamin D supplementation can reduce the number of virus particles that could attach to the ACE2 receptors and enter the cell by promoting the binding of the SARS-CoV-2 cell entry receptor ACE2 to AGTR1 (angiotensin II receptor type 1) (196). Altogether, although these data show that vitamin D can act at different stages of the immune response, administration of high doses of this vitamin as a therapy should be done under medical control mainly for individuals with diseases or disorders.

Vitamin A

Vitamin A is represented by many compounds, such as retinol, retinal, and retinoic acid, as well as various provitamin A carotenoids such as α- or β-carotene (197). Vitamin A, naturally found in foods from animal sources, including dairy products, fish, and meat, plays an important role in the regulation of innate and cell-mediated immunity and humoral antibody response (198, 199). The RDA of this micronutrient is 400–900 μg/day (150). A deficiency of vitamin A alters the integrity of mucosal epithelium, such as the eyes, gastrointestinal tract, and the respiratory system, which causes an increase in their susceptibility to many pathogens (199, 200). In fact, it has been shown that deficiency in vitamin A is associated with increased risk of infection (201) and is connected with an increased risk of developing respiratory inflammation and diseases in children (182). Moreover, vitamin A deficiency negatively affects neutrophil, macrophage, NK, and eosinophil cell functions (181, 182, 200, 202). Moreover, a deficiency in vitamin A may promote an excessive inflammatory response by increasing the production of IL-12, thus promoting T cell growth as well as the pro-inflammatory TNF-α, which induces inflammation and potentiates existing inflammatory conditions. Supplementation with vitamin A can reverse these effects (203, 204). Deficiency in this vitamin and its metabolites is also the cause of the alteration of Th1/Th2 balance by decreasing Th2 (200). Furthermore, a study revealed that persons with low vitamin A status showed an increased risk of lung dysfunction and respiratory disease (205). On the other hand, it has been shown that dietary supplementation with vitamin A in humans improves antibody titer response to various vaccines (204). Finally, Imad and colleagues suggested that vitamin A supplementation at 5–20 mg/day, may prevent morbidity and mortality in children from 6 months to 5 years of age (206).

Retinoic acid, the biologically active retinoid metabolite, has been shown to play an important role in the differentiation, maturation, and function of the innate immune system cells (207) and can also activate the NK cells (208). Different pre-clinical and clinical studies have shown that retinoids stimulate secretion and potentiate the effects of IFN-I, which represent a family of cytokines of the early innate immune response to viruses that are being tested against SARS-CoV-2 (209). In this context, it has been proposed that the key mechanism behind the relationship between retinoic acid and IFN-I, is the activation of the retinoic acid-induced gene I (RIG-I), which produces a pattern recognition receptor responsible for sensing RNA viruses, thus playing an important role in early innate anti-viral immune responses (209, 210).

Finally, some carotenoids serve as provitamins or precursors for vitamin A, and may thereby exert immune-modulating functions (196). In fact, it has been shown that carotenoids may regulate membrane fluidity and gap-junction communication (211). Another major factor that makes carotenoids important during the current pandemic is that this family of compounds has the potential to play antiviral roles (212). Furthermore, serum beta-carotene has been significantly associated with reduced risk of death from various diseases including respiratory diseases (213). In the same context, results from one study revealed that higher supplementation of some carotenoids (lutein/zeaxanthin) for people aged 65 years and over was associated with 23% lower respiratory mortality (214). Although the safe total carotenoid recommended intake range between 5.4 and 15.4 mg/day, supplementation with carotenoids should be taken with caution and high doses of β-carotene have been proposed to be prooxidant and toxic (215).

Vitamin E

Vitamin E, a known antioxidant, is found in many foods including vegetable oils, cereals, meat, poultry, eggs, fruits, vegetables, and wheat germ oil. The RDA of this micronutrient is 7–15 mg/day (150). Besides its antioxidant activity, vitamin E is able to optimize and enhance the immune response (181). A diet rich with vitamin E has been shown to protect cell membranes from damage caused by free radicals and support the integrity of epithelial barriers including those of the respiratory system (181). Supplementation with vitamin E, like vitamin A, promotes Th1 cytokine-mediated response accompanied by a decrease in Th2 response. Thus, this supplementation increases lymphocyte proliferation production of IL-2, NK cell cytotoxic activity, as well as the phagocytic activity by alveolar macrophages, which consequently cause an increase in resistance against infectious agents (182). Different studies have shown the effect of vitamin E in preventing infections such as the influenza virus (216). Moreover, a study conducted by Hemila showed that administration of 50 mg/day of vitamin E for 5–8 years may decrease the incidence of pneumonia by 69% in elderly males (217). Similarly, a randomized controlled trial with a total of 617 persons aged at least 65 years showed that a supplementation of 180 mg/day of vitamin E, which is much higher than the RDA, have an effect on lower respiratory tract infections (216).

Vitamin B

Vitamin B is a class of eight water-soluble vitamins that play important roles in cell metabolism. Many food sources are rich in vitamin B, including whole grains, legumes (beans and lentils), seeds and nuts, as well meat (especially liver). All three of these B vitamins are important because they are involved in the intestinal immune system, supporting the gut barrier, which is an important factor in maintaining an efficient immunity, as we will discuss later (218, 219).

Vitamin B6

Vitamin B6 is essential as a co-factor in nucleic acid, amino acid and protein biosynthesis, and therefore is important for proliferation, differentiation, and functioning of immune cells and synthesis of antibodies and cytokines (206, 220). An adequate diet rich in vitamin B should contain an average of 0.6–1.7 mg/day of vitamin B6 (150). Human studies demonstrate that vitamin B6 deficiency not only impairs lymphocyte maturation and growth, even with marginal deficiency, but also lowers the antibody responses as well as reduces responses to mitogens and T-cell activity (182). A deficiency in vitamin B6 also decreases the IL-2 production and NK cell activity and promotes Th2 cytokine-mediated activity, accompanied with a suppression of Th1 (182). It is important to emphasize that an adequate diet rich with vitamin B6 helps to restore cell-mediated immunity and has been shown to improve lymphocyte maturation and growth and increases the number of T-lymphocytes (182). Finally, Cheng and colleagues showed that a daily injection of 50 or 100 mg/day of vitamin B6 increased the immune responses in 51 subjects who stayed in an intensive care unit for over 14 days (221), suggesting that a higher dose than the one suggested by the RDA would have a beneficial effect, supporting the immune system of COVID-19 patients in an intensive care unit.

Vitamin B9 or Folate

Vitamin B9, similar to vitamins B6 and B12, plays an important role in protein synthesis. Therefore, a deficiency in vitamin B9 alters the immune system (165). An adequate diet rich in vitamin B should contain an average of 200–400 μg/day of vitamin B9 (150). A deficiency in vitamin B9 decreases the resistance to infections by inhibiting the proliferation and circulation of CD8+ CTL (221). Moreover, it has been shown that a deficiency in vitamin B9 impairs NK cytotoxicity (182). In this same context, a study including 60 healthy subjects aged over 70 years who received large intakes of vitamin B9 (supplementation of 400 mg/day), showed that the supplemented subjects reported an increase in NK cell cytotoxicity leading to fewer infections, suggesting that vitamin B9 supplementation increased innate immunity and provided protection against infections in elderly people (222).

Vitamin B12

Vitamin B12 is involved in carbon-1 metabolism and interacts with the folate metabolism (223). An adequate diet rich in vitamin B should contain an average of 1.2–2.4 μg/day of vitamin B12 (150). A deficiency in vitamin B12 causes suppression in NK cell activity, a decreased number of lymphocytes, a significant reduction in cells with a role in cell-mediated immunity, and changes in the proportions of CD8+ CTL and CD4+ Th, leading to abnormally high CD4+ Th/CD8+ CTL ratios (182, 219). A study of patients deficient in vitamin B12 showed that a supplementation with vitamin B12 reversed the effects that presented an abnormally high CD4+ Th/CD8+ CTL ratio and suppressed NK cell activity, indicating that this vitamin may act as a modulatory agent for cellular immunity, especially in relation to CD8+ CTL and NK cells (219). It has also been shown that a deficiency in vitamin B12 impairs the antibody response (181). Bunout and colleagues showed that a regular diet including 3.8 μg of vitamin B12 in elderly subjects (aged >70 years) over 4 months increases NK cell cytotoxic activity, leading to increased innate immunity in elderly people (222). Altogether, these studies state the importance of vitamin B12 in maintaining an adequate immune response, especially in older people (aged >65 years) who have low serum B12 concentrations (224).

Vitamin B2 (Riboflavin)

Vitamin B2 has a very important role in many energy-related enzymatic processes (196). The RDA of vitamin B2 is 0.6–1.3 mg/day (150). It has been suggested that vitamin B2 regulates fatty acid oxidation and therefore controls the differentiation and function of immune cells (225).

Vitamin B3 (Niacin)

Vitamin B3 is generally known as nicotinic acid and nicotinamide, which plays an important central role in aerobic respiration. The RDA of vitamin B3 is 8–16 mg/day (150). Vitamin B3 has been shown to modulate the host immune system by inducing the differentiation of Treg (226) and inhibiting the production of the pro-inflammatory cytokines IL-1, IL-6, and TNF-α by macrophages and monocytes (227).

Vitamin B5 (Pantothenic Acid)

Vitamin B5, like some of other B vitamins, is essential in the TCA cycle and fatty acid oxidation (228). The adequate intake (AI) of vitamin B5 is 3–5 mg/day (150). Vitamin B5, similar to vitamin B2, has been shown to be involved in the control of host immunity *via* energy generation by immune cells, which is very important in the case of COVID-19 patients (219).

Vitamin B7 (Biotin)

Vitamin B7 has a crucial role in nutrition and an important effect in immunometabolism. In fact, by being an essential cofactor for acetyl-CoA carboxylase and fatty acid synthase, this vitamin is used by the body to metabolize carbohydrates, fats, and amino acids (229). The AI of vitamin B7 is 12–30 μg/day for adults (150). Vitamin B7 deficiency induces Th1- and Th17-mediated pro-inflammatory responses in human CD4+ T lymphocytes (230). In the same context, a diet rich in vitamin B7 has anti-inflammatory effects and inhibits the activation of the transcription of NF-κB and thus inhibits the secretion of pro-inflammatory cytokines such as TNF-α, IL-1, IL-6, and IL-8 (231).

Minerals

Zinc

Whole grains, milk products, oysters, red meat, and poultry are good sources of zinc, and the RDA of this micronutrient is between 2 and 11 mg/day (150). Zinc is an essential micronutrient required for controlling key biological processes, and is involved in the regulation of both the innate and adaptive immune system (222). Zinc-deficient subjects may show severe disturbances in immune cell numbers and activities and may experience increased susceptibility to a variety of pathogens (222). Zinc is important for the structural and functional integrity of the skin and mucosal cells (189). Zinc-deficiency is manifested

by an increased thymic atrophy, an imbalance in the Th1/Th2 ratio, characterized by a reduction in Th1 cell numbers, a decrease in lymphocyte proliferation and function, particularly T cells, and alteration in cytokine production—all of these contributing to greater oxidative stress and inflammation (181, 182). Zinc deficiency also impairs survival, proliferation, and maturation of monocytes, NK cells, T and B cells, phagocytosis by macrophages and neutrophils, as well as antibody responses to T cell-dependent antigens (181, 182). It has been shown that correction of zinc deficiency boosts the defense-related immune system, and reduces mortality from infectious diseases and viral infections (222, 232). From several controlled studies, it is clear that daily dietary supplementation of zinc for the elderly and children at high risk for zinc deficiency, is protective against infection and is associated with a decrease in mortality from infections in these populations (233–237). Furthermore, persons with a low zinc status have showed an increased risk of viral infections (238). A systematic review and a metanalysis study showed that zinc at doses of at least 75 mg/day is able to significantly reduce the duration of symptoms caused by viral infection on the upper respiratory tract but does not consistently improve the overall severity of symptoms (239).

Iron

This micronutrient is present in animal sources such as red meat and poultry, as well as in plants such as beans and lentils, cashews, spinach, and whole grains. It is important to note that the body absorbs two to three times more iron from animal sources than from plants. Iron is an essential micronutrient for the differentiation and growth of epithelial tissue as a first line of defense against pathogens (189). A diet rich in iron (10–18 mg/day) (150), or iron dietary supplementation, improves intracellular microbial killing and cellular immunity by forming toxic hydroxyl radicals, and is thus involved in the killing of pathogens by neutrophils and maintaining a certain level of lymphocyte bactericidal activity (189). Iron also has an important role in maintaining a certain level of IL-6 and IFN-γ production, as well as in the differentiation and the proliferation of T cells and in helping to regulate the ratio between CD4+ Th and CD8+ CTL (189). It has been shown that iron supplementation in children reduces the risk of respiratory tract infection (182). On the other hand, high doses of iron leads to increased viral mutations in the influenza virus genome resulting in a more virulent phenotype (240).

Magnesium

This micronutrient is present in greens, nuts, seeds, dry beans, whole grains, and low-fat dairy products. An adult diet containing 320–420 mg/day of magnesium can decrease oxidative stress by reducing the superoxide anion production, protecting the cells from oxidative damage (182). Magnesium also boosts the immune system by increasing NK-cell activity, regulating leukocyte activity and the ratio between CD4+ Th and CD8+ CTL, decreasing the levels of cytokines such as IL-6, and decreasing inflammation (182). Finally, it is important to note that magnesium is involved in antibody responses through antibody—particularly IgG—production, which is important in maintaining immune tolerance in order to distinguish between the "self" and the "non-self" (241).

Selenium

Among the nutrients implicated in viral infection, selenium is a nutritional antioxidant incorporated as a rare amino acid selenocysteine in selenoproteins (242). The RDA of this micronutrient is between 15 and 55 μg/day (150). Selenium plays an important role in antioxidant defense, by regulating reactive oxygen species (ROS) and redox status in tissues. Dietary selenium strongly influences inflammation and immune responses. Some *in vitro* studies on influenza showed that selenium deficiency resulted in reduced antioxidant activity of cells and an important increase in the pro-inflammatory cytokine IL-6, altering the response to influenza of epithelial cells (242). In addition, studies by Beck et al. (243, 244) showed that host selenium deficiency increased the virulence of RNA viruses such as coxsackievirus B3 and influenza A (242), while pointing at an interesting endemic disease in the northeast of China, where soil is selenium-deficient, namely Keshan disease. This disease is interesting to relate, as it is a seasonal cardiomyopathy for which the virus coxsackievirus B3 was identified as being a co-factor (243–245). Interestingly, when the population received a supplementation in selenium, the incidence of the disease decreased dramatically. In addition, selenium prevented mutations of the viral genomic RNA that lead to increased virulence and cardiac pathology (242). Finally, selenium was shown to be associated with a decrease in the occurrence of ventilator associated pneumonia in mechanically ventilated patients (246).

Iodine

It is well-known that a large number of people around the world do not consume enough iodine (247). However, deficiency is rare in developed countries because of iodized salt. The RDA of iodine is 150 μg/day for both males and females over 14 years old, while it increases to 220 μg/day during pregnancy and to 290 μg/day during breastfeeding (150). It has been shown that iodine presents a role in modulating the function of human immune cells and present some therapeutic effects in different pathologies (248, 249). A study showed that iodine is able to increase the movement of granulocytes into the area of inflammation and to improve their ability for phagocytosis, clearing infections (249). Furthermore, it has been reported that iodine has an indirect effect on the modulation of the immune system by modulating the thyroid hormone synthesis (248). The modulation of the thyroid hormones enhances NK cytotoxicity, the expression of cytokines as well as B cell differentiation and increases the frequency of T memory cells (248).

Copper

While enough dietary copper can be obtained from solids and water, it is important to mention the effect of copper deficiency, as it can occur in seriously ill individuals who require parenteral nutrition. The RDA of copper is 440–900 μg/day (150). Copper deficiency can also occur in older people as a result of malnutrition or malabsorption. Failure to correct this

might lead to susceptibility to further infections by decreasing the number of circulatory blood cells (182, 250–252). Recent studies supported the role for Cu in the innate immune response against infections (250). Raha et al. hypothesized that copper supplementation can help to fight COVID19, especially in older people where a deficiency of Cu is a strong possibility (250). In fact, they suggested that a diet supplemented with Cu affects host immune function and metabolism of other micronutrients, prevents the severity of the viral infection and may protect people from COVID-19 (250). Finally, it is important to note that a wide array of lung infections can be accompanied by elevated copper levels (253) and that an accumulation of copper can also be toxic (254, 255).

Polyphenols

Polyphenols are produced in plants and can be classified into flavonoids, phenolic acids, polyphenolic amides, and other compounds (256). In addition to their well-established anti-inflammatory and anti-oxidant activities, studies have highlighted their antiviral potential. For example, antiviral properties of some polyphenols have been demonstrated against several viruses including Epstein-Barr, enterovirus, herpes simplex, and influenza (257). However, only a limited number of studies have investigated the role of polyphenols against coronaviruses directly (257). We will briefly cite the important polyphenols that have been tested in this regard. Ten polyphenolic compounds isolated from *Brussonetia* papyrifera proved effective against MERS/SARS-CoV proteases (258). Ethanolic extracts of Sambucus formosana proved effective against the human coronavirus strain HCoV-NL63 (259). Saikosaponin B2 has also shown good potency in this regard (258). Griffithsin is a polyphenol extracted from a red algae called Griffithsia genus and is one of the most promising inhibitors of MERS-CoV (258). By specifically binding to glycans of the CoV protein spikes, it can inhibit attachment of the virus to host cells, with high potency, making this polyphenol a good candidate for trials against SARS-CoV-2. Silvestrol is another polyphenol compound, extracted from *Aglaia* sp., that showed inhibitory properties against MERS-CoV (258).

Resveratrol (RSV) is probably the most promising polyphenol to test against SARS-CoV2. Indeed, it has been found to significantly inhibit MERS-CoV RNA replication *in vitro* on Vero E6 cells, *via* several mechanisms including inhibition of the virus protein expression, inhibition of the NFκB pathway and activation of the AMPK/Sirt1 axis in the host cell (257). RSV is found in mulberries, grapes, red wine, and peanuts, and was showed to possess—in addition to its antiviral properties—antioxidant, antitumoral effects, and scavenger of free radicals properties (260). A study tried to add RSV to the diet of piglets exposed to rotavirus and showed that RSV decreased TNF-α levels and diminished diarrhea in a resveratrol piglet diet (261). Another interesting study demonstrated the ability of RSV to counteract MERS-CoV infection by acting at different levels from reducing the cell death, inhibiting the viral replication, reducing the viral titer and inhibiting the expression of the nucleocapsid proteins, as well as inhibiting the apoptosis. This study demonstrates that RSV can be an adjunctive antiviral agent to consider in testing against SARS-CoV2. Finally a new clinical trial has been registered in the database clinicaltrial.gov to test the effect of resveratrol on COVID19 patients (NCT04400890) (262).

Although, data suggest that micronutrients play an important role in strengthening the immune system, it must be emphasized that the body requires optimal levels of micronutrients for effective immune function, with different requirements throughout every stage of life. For this reason, it is important to be aware that RDA for all nutrients is the *average* daily requirement necessary to avoid clinical or subclinical deficiency in the majority of people (97–98%) in a healthy general population (**Table 3**) (263). These RDA can be lower than effective therapeutic recommended doses needed to increase immune system responses in order to fight viral infections.

ROLE OF PROBIOTICS, DIET AND FASTING IN IMMUNE FUNCTION

The Role of Probiotics in Immune Function

According to the FDA and the WHO, probiotics are defined as "live micro-organisms which can provide health benefits on the host when administered in adequate amounts" (264). Ever since probiotics were recognized for their beneficial effects on health, they have been used as potential dietary supplements (265). Probiotics or the gut bacteria produce various metabolites and co-metabolites as by-products of food metabolism (266). These molecules, produced by the gut microbiota, have the ability to cross the gut-blood barrier and affect the health through various mechanisms, such as energy supplementation for colonic epithelium and anti-inflammatory activity (267). One of the most important groups of metabolites produced by the gut microbiota through undigested fermented food are SCFAs (discussed in a previous section), such as acetic acid, butyric acid, propionic acid, that have been shown to have a beneficial effect by maintaining the integrity of the epithelial barrier, decreasing the "leaky gut," and, as a consequence, triggering an inflammatory reaction and the modulation of oxidative stress and the immune response (268). In fact, probiotics are able to modulate the immune and the inflammatory response in the gut through their interaction with the gut mucosa and mucosal immune system, which host the largest part of the body's immune cells mainly within the gut-associated lymphoid tissue (263). Various studies have shown that probiotics are able to induce both: (1) the production of pro-inflammatory cytokines in order to facilitate the immune system against a further infection, and (2) the production of anti-inflammatory cytokines in order to have a balanced homeostasis by reducing an excessive inflammatory reaction induced by an infection (263). Moreover, probiotics' health benefits are not only limited to the intestinal tract, but also present modulatory effects in other locations of the mucosal system, such as the upper respiratory tract (269). In the same context, it has been shown that besides infecting the respiratory tract, SARS-CoV-2 can also infect the lower gastrointestinal tract, which is rich in ACE2 receptors (270).

Probiotics can have an effect on both the innate immune system and the adaptive immune system. Some probiotics

TABLE 3 | Recommended dietary allowance.

Macronutrients and micronutrients	Recommended dietary allowance		
	Children, M/F 4–8 years 9–13 years 14–18 years	Adults, M/F 19–50 years	Old age, M/F 51–>70 years
Fats, g/day	ND	ND	ND
Carbohydrates, g/day	130 130 130	130	130
Proteins, g/day	19 34 52	34/56	46/56
Vitamin C, mg/day	25 45 65/75	75/90	90/75
Vitamin D, μg/day	15	15	15/20
Vitamin A, μg/day	400 600 700/900	700/900	700/900
Vitamin E, mg/day	7 11 15	15	1.5/1.7
Vitamin B6, mg/day	0.6 1 1.2/1.3	1.3	1.5/1.7
Vitamin B12, μg/day	1.2 1.8 2.4	2.4	2.4
Vitamin B9, μg/day	200 300 400	300/400	400
Vitamin B2, mg/day	0.6 0.9 1.3	1.1/1.3	1.1/1.3
Vitamin B3, mg/day	8 12 16	14/16	14/16
Vitamin B5, mg/day	3* 4* 5*	5*	5*
Vitamin B5, μg/day	12* 20* 25*	30*	30*
Zinc, mg/day	5 8 11/9	8/11	8/11
Iron, mg/day	10 8 11/15	8/11	8
Magnesium, mg/day	130 240 360/410	310/420	420/320
Selenium, mg/day	30–40	55–70	55–70
Copper, mg/day	900–1,100	1,400–1,700	1,400–1,700
Iodine, mg/day	90–120	150	150

Except vitamin B5 and vitamin B7 where the values followed by an asterisk (*) represent the AIs, the values related to other micronutrients and micronutrients present the RDAs.

achieve this beneficial effect by acting on the mucosal immune system, in particular DCs and NK cells (271). As an example, it has been shown that administration of *lactobacilli* to mice can enhance the immune function in mice by increasing NK cell activity and phagocytic activity of macrophages (272), as well as enhance the phagocytic capacity of peritoneal leukocytes (273), increase the expression of DC-maturation markers, and enhance lymphocyte proliferation (274). Consistent with studies using animal models, human studies also showed that probiotic use could have a positive effect on the immune system. Healthy, older individuals receiving *Lactobacillus rhamnosus* HN001 or *Bifidobacterium lactis* HN019 in a milk-based diet showed increases in their peripheral blood proportion of NK cells and their tumoricidal activity, as well as increases in phagocytic activity (275). Another study showed that a daily ingestion of fermented milk containing *Lactobacillus casei* DN114001 improved innate-defense capacity in 45 healthy, middle-aged people (aged 51–58 years) by increasing the oxidative burst capacity of monocytes as well as NK cells' tumoricidal activity (276).

There is also evidence that supplementation with probiotics has beneficial effects on the adaptive immune system by modulating the functions of both T and B cells while preventing an autoimmune inflammatory response (263). The effects of probiotics on T cells varies widely depending on the strain, going from promoting the production of Th1 (IFN-γ, IL-2, IL-12, TNF-α), Th17 (IL-17, IL-22), and Treg (IL-10, TGF-β) cytokines, to the inhibition of Th2 cytokines (IL-4) (208, 277). In animal studies, the administration of *Bifidobacterium bifidum* (5×10^8 CFU/d) for 8 week for old mice, showed an enhancement of anti-oxidation activity in the thymus and spleen, alteration of gene expression, and improvement in immune function, leading to significantly increased cytokine IL-2 and IFN-γ levels but also decreased pro-inflammatory cytokines IL-6 and TNF-α concentrations (278). Mane and colleagues showed that the consumption of a skim milk rich with a mixture of *Lactobacillus plantarum* CECT 7315 and CECT 7316 for 12 weeks, enhanced systemic immunity in elderly subjects, manifested by fewer incidences of infection and mortality due to pneumonia, compared to those who received unenriched skim milk only (279). The study showed that the participants who consumed the skim milk enriched with probiotics had increased percentages of B cells, NK cells, CD4+, and CD8+ and that most of these changes lasted for another 12 weeks after stopping the consumption of the probiotics (279). Guillemard and colleagues conducted a double blind, controlled study, involving 1,072 volunteers (median age = 76.0 years) who were given a fermented dairy product containing the probiotic *Lactobacillus casei* DN-114001 (280). This study showed that supplementation with the fermented product was safe and was associated with a decrease in the duration of respiratory infections in comparison with the control group (280). A similar study showed that the consumption of yogurt fermented with *L. bulgaricus* OLL1073R-1, augmented NK cell activity and reduced the risk of infection and the risk of catching the common cold in elderly individuals (281). Altogether these studies suggest that the administration of

probiotics can enhance the host's resistance against infection for older subjects and reduce the severity of viral infection in both the gastrointestinal tract and the respiratory tract.

Like probiotics, some selective prebiotics—which is defined as a substrate that is selectively utilized by host microorganisms conferring a health benefit—have also been reported to be beneficial for health. In this context, most of the studies considered that prebiotics have indirect effects on the immune system through changing the composition and population of gut microbiota (282). It has been shown that prebiotic compounds such as inulin, polydextrose, and maize fiber are able to improve the immune response, gut diversity, and digestion in humans—especially in elderly people (283, 284). In addition to the effects on the composition of the microbiota, prebiotics also produce notable shifts in the immune system by increasing the expression of anti-inflammatory cytokines, while reducing the expressions of pro-inflammatory cytokines (285, 286). Also, it is known now that prebiotics such as wheat bran, fructo-oligosachharides, and galactosachharides are known to increase butyrate levels thereby reducing inflammation and improving conditions in asthma and cystic fibrosis (287). It is to be noted that beneficial effects of the prebiotics are thought to be mediated mostly by increased production of SCFAs and strengthening of the gastrointestinal immune system. Overall, it is apparent that diet mediated modulation of gut microbiota, and to some extent even lung microbiota, can influence immunity and reduce the severity of viral infection in both the gastrointestinal tract and the respiratory tract (270, 287).

Taking into consideration that probiotics and prebiotics are generally safe, this microbiome therapy may improve and quicken the recovery of elderly patients and immune-compromised COVID19 patients. We suggest that probiotics/prebiotics that have been shown to have antiviral and respiratory benefits can be used as part of the actual therapies used to reduce infection with SARS- CoV-2. Nutritional recommendations could include a combinations of pre and probiotics (symbiotic), such as fructo-oligosaccharides and galactosaccharides, and various lactobacilli strains to improve gut dysbiosis, thereby improving the overall immune response (270, 288).

Diet and Fasting

The health effects of various forms of fasting have been studied for decades and the database clinicaltrials.gov currently has 1,901 trials registered under the MeSH term "fasting" for a large array of diseases and disorders. Water fasting (which restricts everything except water), intermittent 16 h fasting, the fasting mimicking diet (FMD), and religious "Ramadan" fasting are the most common types of fasting under study. In particular, it is important to highlight the concurrent COVID19 pandemic with this year's "Ramadan" fasting. This is important because Islam has 1.8 billion adherents, the majority of whom were fasting during the pandemic. As this situation is highly unusual, many questions were raised as to whether fasting during the pandemic is safe or not. This situation has led physicians and scientists to consider the risks and benefits of fasting for their patients during the pandemic. This exceptional situation shows promise in providing data for observational clinical studies which will be shown progressively in future scientific literature (289–291). For these reasons, we will briefly review the risks and benefits of fasting during the COVID-19 pandemic.

A study in this regard conducted by Develioglu and colleagues revealed that lymphocyte numbers increased significantly, that serum IgG and salivary IgA decreased and that there were no changes in serum IgM (292). In fact, some evidence suggests that "Ramadan" fasting can actually change the functions of the immune system (291, 293). Other studies have shown the beneficial effect of intermittent, prolonged fasting during the month of Ramadan and how this could affect the inflammatory state (293–296). An investigation of 50 healthy volunteers who practiced "Ramadan" fasting was conducted 1 week before "Ramadan" fasting, at the end of the third week of "Ramadan," and 1 month after the cessation of "Ramadan" (293). In this study, the authors showed that intermittent Ramadan fasting for a month, attenuated pro-inflammatory cytokines (IL-1β, IL-6) and decreased the number of lymphocytes, neutrophils, and monocytes in circulation as well as decreased the abdominal fat in healthy subjects (293). Similarly, another study on fasting for 1 month examined the effect of this prolonged intermittent fasting on serum cytokines levels in healthy and obese individuals (295). This study showed that the levels of different inflammatory biomarkers, including serum white blood cells (WBCs), IL-2, IL-8, and TNF-α, were significantly lower in both the control group and the obese group in comparison to pre-Ramadan values (295). Although these two studies showed that immune cells decreased during Ramadan but remained within the reference ranges, much more data are needed on this topic.

A recent study revealed that fasting can be quite safe for normal healthy individuals and can lead to "some beneficial changes in some inflammatory markers, as well as metabolic measurements" (297). Results showed decreased levels of pro-inflammatory chemokines GRO (growth-regulated oncogene)-alpha (Gro-α), IP-10, and stromal cell-derived factor 1 (SDF-1) in comparison with cytokine and chemokine profiles of COVID-19 patients that show marked elevation (298).

Furthermore, another study demonstrated that prolonged intermittent fasting has some positive effects on the inflammatory status (296). This study showed not only that the level of IL-6 decreased during fasting but the data also showed increases in circulating levels of vitamin B12 and folate, which have been previously found to be beneficial in supporting the immune system against viral infection. Another study showed that Ramadan fasting does not alter oxidative stress parameters or biochemical markers of cellular damage in healthy subjects. Although this study revealed a decrease in the level of carotenoids, which has previously been shown to exert immune-modulating functions (196), a slight reduction in lipid peroxidative damage in erythrocytes and no changes in retinol, vitamin E, and C have been observed (299). In fact, oxidative stress has been shown to be implicated on viral pathogenesis and infections (300, 301) and reducing lipid peroxidative damage in erythrocytes may reduce the consequences of viral infection.

It has been shown that fasting can decrease immunosenescence, extend life expectancy (302), reduce markers of oxidative stress and inflammation, and improve lung function, as well as to alleviate or reverse autoimmune disorders (303–305). The other studies on Ramadan fasting also showed reduced immune cell numbers, even though some found no changes (301). Only one study (292) on a small number of male subjects showed increased lymphocyte numbers.

Although some of the discussed results may support the hypothesis that fasting during the pandemic lockdown might not have a negative effect and might actually support the immune system response in case of an infection by SARS-CoV2, much more data are needed on this topic. One recent review and systematic analysis on the effects of Ramadan fasting on immunity by Adawi et al. (306) showed that the effects were diverse, and that the study samples were small, thus, a definite conclusion cannot be made.

CONCLUSION

Nutrition and diet are able to promote the functioning of the immune system as a preventive measure by reducing both inflammation and oxidative stress that might be caused by various factors. Deficiencies in some micronutrients can increase inflammation and the risk of infection (196). Several of the micronutrients discussed in this review, can interact with transcription factors to regulate the expression of receptors used by viruses such as ACE2 (196). In addition, nutrition and diets modulate the gut microbiota, which can affect gut permeability and inflammatory status.

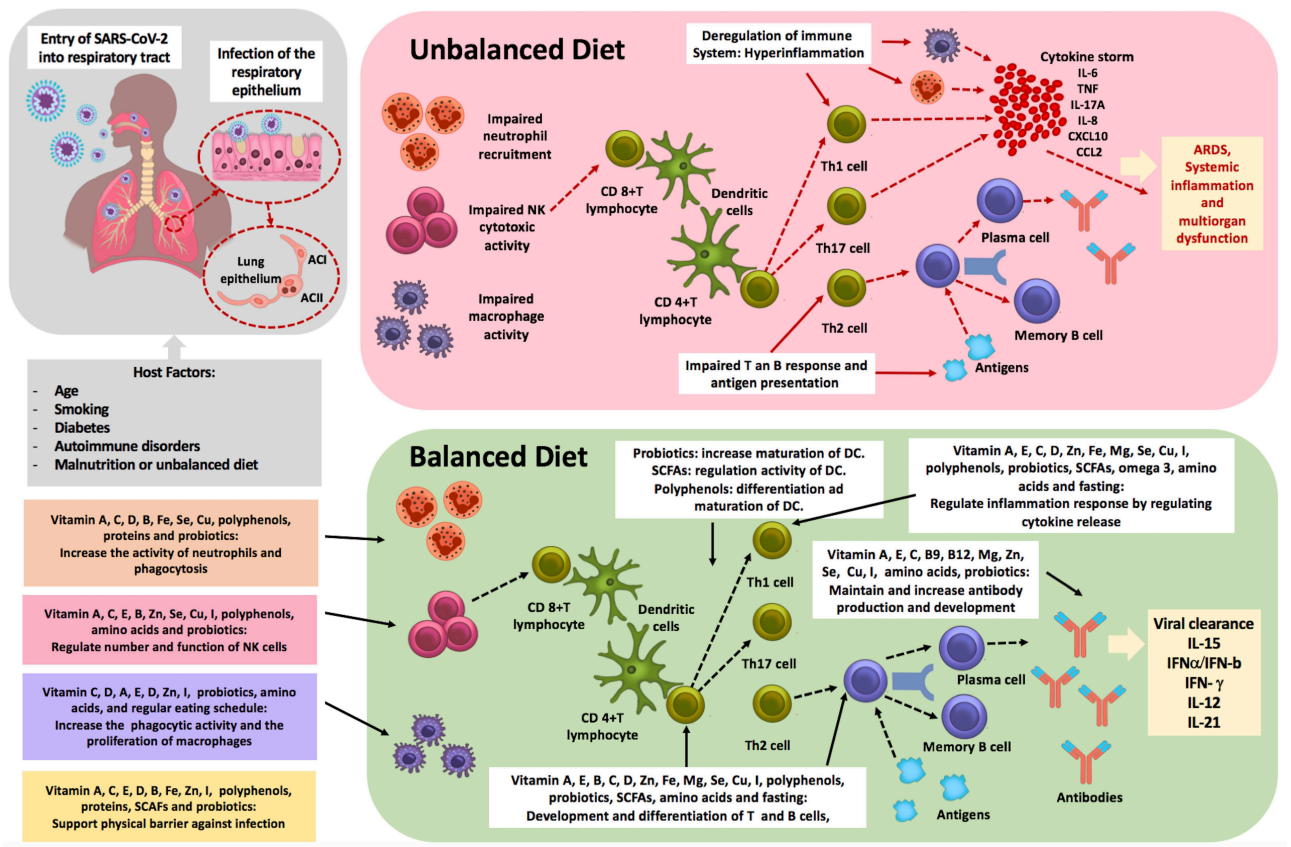


FIGURE 2 | Important role of nutrition in strengthening the immune system in regard to the fight against SARS-CoV-2 infection. Red box: The effect of an unbalanced diet on the immune system response. Different host factors including age, smoking, diabetes, autoimmune disorders, malnutrition, or an unbalanced diet may affect the immune system response, leading to high levels of inflammation which explain the severe cases of COVID-19. In fact, in this case, invasion of the respiratory epithelium and other target cells by SARS-CoV-2 involves T-lymphocytes infection and apoptosis, leading to their decreased number and activity, and the consecutive impaired activation of B cells and the production and secretion of antibodies. This leads to the compensatory increased neutrophil and macrophage activity, their accumulation in the lungs and hyper-secretion of cytokines, in order to re-activate the adaptive immune system. The viral clearance is delayed and prolonged infection causes a decrease in ACE2 receptors, leading to over-activity of renin-angiotensin II system (RAS), which causes endothelial dysfunction and thrombosis. This could lead to a cytokine storm, accompanied by Respiratory Distress Syndrome (ARDS) and multiorgan dysfunction—characteristics of severe cases of COVID-19. Green box: The effect of a balanced diet on the immune system response. Vitamins A, C, D, B, E, iron, magnesium, zinc, copper, iodine, selenium, proteins, SCFAs, omega-3, a low-carb diet, polyphenols, probiotics, and a balanced diet were shown to directly support the body's natural defense system by enhancing the different levels of immunity and, therefore, might participate in the development of a strong immune system, which may help the body's immune system fight any viral infection and promote virus clearance.

It is essential that probiotics and necessary nutrients such as vitamins—which affect the immune system—are not neglected before and during infection. Vitamins A, C, D, B, E, iron, magnesium, zinc, copper, selenium, iodine, proteins, SCFAs, omega-3, a low fat diet, and polyphenols were shown to directly support the body's natural defense system by enhancing the different levels of immunity and therefore might promote virus clearance (**Figure 2**). It follows that infected patients who already have nutritional deficiencies or excess may have an inadequate inflammatory reaction causing more severe negative clinical outcomes.

Future clinical studies should not neglect the potential of minerals, vitamins, polyphenols, and probiotics in modulating the immune response (307). Moreover, close monitoring of micronutrient levels during treatment of COVID19 patients would contribute to a great advance in understanding the role of nutrition in treatment of COVID19.

AUTHOR CONTRIBUTIONS

AC conceived the study, researched, and wrote the manuscript. CM helped in editing the text and sketched the figures. GB and DZ helped in writing part of the manuscript and editing the text. All authors contributed to the proofreading of the manuscript and approved the final version of the manuscript.

FUNDING

The publication of this research was supported by the premedical Department at Weill Cornell Medicine Qatar.

ACKNOWLEDGMENTS

I acknowledge the help of Sinead M. O'Rourke, Weill Cornell Medicine-Qatar Distributed e-Library, for editing the manuscript.

REFERENCES

1. Petrosillo N, Viceconte G, Ergonul O, Ippolito G, Petersen E. COVID-19, SARS and MERS: are they closely related? *Clin Microbiol Infect.* (2020) 26:729–34. doi: 10.1016/j.cmi.2020.03.026
2. Guarner J. Three emerging coronaviruses in two decades: the story of SARS, MERS, and now COVID-19. *Am J Clin Pathol.* (2020) 153:420–1. doi: 10.1093/ajcp/aqaa029
3. Wu D, Wu T, Liu Q, Yang Z. The SARS-CoV-2 outbreak: what we know. *Int J Infect Dis.* (2020) 94:44–48. doi: 10.1016/j.ijid.2020.03.004
4. Muniyappa R, Section CE, Branch O, Diseases K. COVID-19 pandemic, coronaviruses, and diabetes mellitus. *Am J Physiol Endocrinol Metab.* (2020) 318:E736–41. doi: 10.1152/ajpendo.00124.2020
5. Li C, Yang Y, Ren L. Genetic evolution analysis of 2019 novel coronavirus and coronavirus from other species. *Infect Genet Evol.* (2020) 82:1–3. doi: 10.1016/j.meegid.2020.104285
6. Nelemans T, Kikkert M. Viral innate immune evasion and the pathogenesis of emerging RNA virus infections. *Viruses.* (2019) 11:961. doi: 10.3390/v11100961
7. Fung SY, Yuen KS, Ye ZW, Chan CP, Jin DY. A tug-of-war between severe acute respiratory syndrome coronavirus 2 and host antiviral defence: lessons from other pathogenic viruses. *Emerg Microbes Infect.* (2020) 9:558–70. doi: 10.1080/22221751.2020.1736644
8. Mehta P, McAuley DF, Brown M, Sanchez E, Tattersall RS, Manson JJ. COVID-19: consider cytokine storm syndromes and immunosuppression. *Lancet.* (2020) 395:1033–4. doi: 10.1016/S0140-6736(20)30628-0
9. Zhang J, Wang X, Jia X, Li J, Hu K, Chen G, et al. Risk factors for disease severity, unimprovement, and mortality in COVID-19 patients in Wuhan, China. *Clin Microbiol Infect.* (2020) 26:767. doi: 10.1016/j.cmi.2020.04.012
10. Munster VJ, Koopmans M, van Doremalen N, van Riel D, de Wit E. A novel coronavirus emerging in China - key questions for impact assessment. *N Engl J Med.* (2020) 382:692–4. doi: 10.1056/NEJMp2000929
11. Liu H, Chen S, Liu M, Nie H, Lu H. Comorbid chronic diseases are strongly correlated with disease severity among COVID-19 patients: a systematic review and meta-analysis. *Aging Dis.* (2020) 11:668. doi: 10.14336/AD.2020.0502
12. Furukawa NW, Brooks JT, Sobel J. Evidence supporting transmission of severe acute respiratory syndrome coronavirus 2 while presymptomatic or asymptomatic. *Emerg Infect Dis.* (2020) 26:6059. doi: 10.3201/eid2607.201595
13. Qin C, Zhou L, Hu Z, Zhang S, Yang S, Tao Y, et al. Dysregulation of immune response in patients with COVID-19 in Wuhan, China. *Clin Infect Dis.* (2020) 71:762–8. doi: 10.1093/cid/ciaa248
14. Thevarajan I, Nguyen THO, Koutsakos M, Druce J, Caly L, van de Sandt CE, et al. Breadth of concomitant immune responses prior to patient recovery: a case report of non-severe COVID-19. *Nat Med.* (2020) 26:453–5. doi: 10.1038/s41591-020-0819-2
15. Caso F, Costa L, Ruscitti P, Navarini L, Del Puente A, Giacomelli R, et al. Could Sars-coronavirus-2 trigger autoimmune and/or autoinflammatory mechanisms in genetically predisposed subjects? *Autoimmun Rev.* (2020)19:102524. doi: 10.1016/j.autrev.2020.102524
16. Li G, Fan Y, Lai Y, Han T, Li Z, Zhou P, et al. Coronavirus infections and immune responses. *J Med Virol.* (2020) 92:424–32. doi: 10.1002/jmv.25685
17. Christian LM, Glaser R. The impact of everyday stressors on the immune system and health. In: *Stress Challenges and Immunity in Space: From Mechanisms to Monitoring and Preventive Strategies* (2012). p. 31–43.
18. Shaha M, Roy B, Akter T, Karim E, Rahaman M. Host immune responses to the infections caused by the infectious viruses. *Acta Sci Microbiol.* (2018) 1:13–6.
19. Bae YS, Shin EC, Bae YS, Van Eden W. Editorial: stress and immunity. *Front Immunol.* (2019) 10:245. doi: 10.3389/fimmu.2019.00245
20. Prompetchara E, Ketloy C, Palaga T. Immune responses in COVID-19 and potential vaccines: lessons learned from SARS and MERS epidemic. *Asian Pacific J Allergy Immunol.* (2020) 38:1–9. doi: 10.12932/AP-200220-0772
21. Calder PC, Carr AC, Gombart AF, Eggersdorfer M. Optimal nutritional status for a well-functioning immune system is an important factor to protect against viral infections. *Nutrients.* (2020) 12:1181. doi: 10.3390/nu12041181
22. Briguglio M, Pregliasco FE, Lombardi G, Perazzo P, Banfi G. The malnutritional status of the host as a virulence factor for new coronavirus SARS-CoV-2. *Front Med.* (2020) 7:146. doi: 10.3389/fmed.2020.00146
23. Katona P, Katona-Apte J. The interaction between nutrition and infection. *Clin Infect Dis.* (2008) 46:1582–8. doi: 10.1086/587658
24. Oz HS. Nutrients, infectious and inflammatory diseases. *Nutrients.* (2017) 9:1085. doi: 10.3390/nu9101085
25. Somerville VS, Braakhuis AJ, Hopkins WG. Effect of flavonoids on upper respiratory tract infections and immune function: a systematic review and meta-analysis. *Adv Nutr.* (2016) 7:488–97. doi: 10.3945/an.115.010538
26. Pérez-Cano FJ, Castell M. Flavonoids, inflammation and immune system. *Nutrients.* (2016) 8:8–11. doi: 10.3390/nu8100659
27. Dobner J, Kaser S. Body mass index and the risk of infection - from underweight to obesity. *Clin Microbiol Infect.* (2018) 24:24–8. doi: 10.1016/j.cmi.2017.02.013
28. Philad Abbas AK. Lichtman HA, Pillai S. *Basic Immunology*, 6th ed (2019).
29. Embgenbroich M, Burgdorf S. Current concepts of antigen cross-presentation. *Front Immunol.* (2018) 9:1643. doi: 10.3389/fimmu.2018.01643

30. Theoharides TC, Alysandratos KD, Angelidou A, Delivanis DA, Sismanopoulos N, Zhang B, et al. Mast cells and inflammation. *Biochim Biophys Acta Mol Basis Dis.* (2012) 1822:21–33. doi: 10.1016/j.bbadis.2010.12.014
31. Prame Kumar K, Nicholls AJ, Wong CHY. Partners in crime: neutrophils and monocytes/macrophages in inflammation and disease. *Cell Tissue Res.* (2018) 371:551–65. doi: 10.1007/s00441-017-2753-2
32. Stone KD, Prussin C, Metcalfe DD. IgE, mast cells, basophils, and eosinophils. *J Allergy Clin Immunol.* (2010) 125(2 Suppl. 2). doi: 10.1016/j.jaci.2009.11.017
33. Vivier E, Tomasello E, Baratin M, Walzer T, Ugolini S. Functions of natural killer cells. *Nat Immunol.* (2008) 9:503–10. doi: 10.1038/ni1582
34. Lodoen MB, Lanier LL. Natural killer cells as an initial defense against pathogens. *Curr Opin Immunol.* (2006) 18:391–8. doi: 10.1016/j.coi.2006.05.002
35. Hoeksema MA, Scicluna BP, Boshuizen MCS, van der Velden S, Neele AE, Van den Bossche J, et al. IFN-γ priming of macrophages represses a part of the inflammatory program and attenuates neutrophil recruitment. *J Immunol.* (2015) 194:3909–16. doi: 10.4049/jimmunol.1402077
36. Atri C, Guerfali FZ, Laouini D. Role of human macrophage polarization in inflammation during infectious diseases. *Int J Mol Sci.* (2018) 19:1801. doi: 10.3390/ijms19061801
37. Ma WT, Yao XT, Peng Q, Chen DK. The protective and pathogenic roles of IL-17 in viral infections: friend or foe? *Open Biol.* (2019) 9:190109. doi: 10.1098/rsob.190109
38. Veiga-Parga T, Sehrawat S, Rouse BT. Role of regulatory T cells during virus infection. *Immunol Rev.* (2013) 255:182–96. doi: 10.1111/imr.12085
39. Murin CD, Wilson IA, Ward AB. Antibody responses to viral infections: a structural perspective across three different enveloped viruses. *Nat Microbiol.* (2019) 4:734–47. doi: 10.1038/s41564-019-0392-y
40. Tay MZ, Wiehe K, Pollara J. Antibody dependent cellular phagocytosis in antiviral immune responses. *Front Immunol.* (2019) 10:332. doi: 10.3389/fimmu.2019.00332
41. Marshall JS, Warrington R, Watson W, Kim HL. An introduction to immunology and immunopathology. *Allergy Asthma Clin Immunol.* (2018) 14(s2):1–10. doi: 10.1186/s13223-018-0278-1
42. Takeuchi O, Akira S. Innate immunity to virus infection. *Immunol Rev.* (2009) 227:75–86. doi: 10.1111/j.1600-065X.2008.00737.x
43. Zhao J, He S, Minassian A, Li J, Feng P. Recent advances on viral manipulation of NF-κB signaling pathway. *Curr Opin Virol.* (2015) 15:103–11. doi: 10.1016/j.coviro.2015.08.013
44. Chiang H Sen, Liu HM. The molecular basis of viral inhibition of IRF- and STAT-dependent immune responses. *Front Immunol.* (2019) 10:3086. doi: 10.3389/fimmu.2018.03086
45. Lee AJ, Ashkar AA. The dual nature of type I and type II interferons. *Front Immunol.* (2018) 9:2061. doi: 10.3389/fimmu.2018.02061
46. Schoggins JW, Wilson SJ, Panis M, Murphy MY, Jones CT, Bieniasz P, et al. A diverse range of gene products are effectors of the type i interferon antiviral response. *Nature.* (2011) 472:481–5. doi: 10.1038/nature09907
47. Schoggins JW, Rice CM. Interferon-stimulated genes and their antiviral effector functions. *Curr Opin Virol.* (2011) 1:519–25. doi: 10.1016/j.coviro.2011.10.008
48. Teijaro JR. Type I interferons in viral control and immune regulation. *Curr Opin Virol.* (2016) 16:31–40. doi: 10.1016/j.coviro.2016.01.001
49. Swiecki M, Colonna M. Type I interferons: diversity of sources, production pathways and effects on immune responses. *Curr Opin Virol.* (2011) 1:463–75. doi: 10.1016/j.coviro.2011.10.026
50. Muraille E, Leo O, Moser M. Th1/Th2 paradigm extended: macrophage polarization as an unappreciated pathogen-driven escape mechanism? *Front Immunol.* (2014) 5:603. doi: 10.3389/fimmu.2014.00603
51. Shrivastava G, León-Juárez M, García-Cordero J, Meza-Sánchez DE, Cedillo-Barrón L. Inflammasomes and its importance in viral infections. *Immunol Res.* (2016) 64:1101–17. doi: 10.1007/s12026-016-8873-z
52. Wang X, Feuerstein GZ, Gu JL, Lysko PG, Yue TL. Interleukin-1β induces expression of adhesion molecules in human vascular smooth muscle cells and enhances adhesion of leukocytes to smooth muscle cells. *Atherosclerosis.* (1995) 115:89–98. doi: 10.1016/0021-9150(94)05503-B
53. Xia P, Gamble JR, Rye KA, Wang L, Hii CST, Cockerill P, et al. Tumor necrosis factor-α induces adhesion molecule expression through the sphingosine kinase pathway. *Proc Natl Acad Sci USA.* (1998) 95:14196–201. doi: 10.1073/pnas.95.24.14196
54. Korn T, Bettelli E, Oukka M, Kuchroo VK. IL-17 and Th17 Cells. *Annu Rev Immunol.* (2009) 27:485–517. doi: 10.1146/annurev.immunol.021908.132710
55. Schmidt-Arras D, Rose-John S. IL-6 pathway in the liver: from physiopathology to therapy. *J Hepatol.* (2016) 64:1403–15. doi: 10.1016/j.jhep.2016.02.004
56. Subbarayal B, Chauhan SK, Di Zazzo A, Dana R. IL-17 Augments B cell activation in ocular surface autoimmunity. *J Immunol.* (2016) 197:3464–70. doi: 10.4049/jimmunol.1502641
57. Lunding L, Vock C, Webering S, Behrends J, Hölscher C, Fehrenbach H, et al. The role of IL-17 producing NK cells and other innate immune cells in poly(I:C) triggered exacerbation of experimental asthma. *Eur Respir J.* (2016) 48:PA1102. doi: 10.1183/13993003.congress-2016.PA1102
58. O'Brien RL, Roark CL, Born WK. IL-17-producing γδ T cells. *Eur J Immunol.* (2009) 39:662–6. doi: 10.1002/eji.200839120
59. Kim G, Gu MJ, Kim SJ, Ko KH, Kye YC, Kim CG, et al. Transcription factor KLF10 constrains IL-17-committed Vγ4+ γδ T cells. *Front Immunol.* (2018) 9:196. doi: 10.3389/fimmu.2018.00196
60. Lawand M, Déchanet-Merville J, Dieu-Nosjean MC. Key features of gamma-delta T-cell subsets in human diseases and their immunotherapeutic implications. *Front Immunol.* (2017) 8:761. doi: 10.3389/fimmu.2017.00761
61. Piccinini AM, Knebl K, Rek A, Wildner G, Diedrichs-Möhring M, Kungl AJ. Rationally evolving MCP-1/CCL2 into a decoy protein with potent anti-inflammatory activity *in vivo*. *J Biol Chem.* (2010) 285:8782–92. doi: 10.1074/jbc.M109.043299
62. Bhavsar I, Miller CS, Al-Sabbagh M. Macrophage inflammatory protein-1 alpha (MIP-1 alpha)/CCL3: as a biomarker. *Gen Methods Biomark Res Appl.* (2015) 1–2:223–49. doi: 10.1007/978-94-007-7696-8_27
63. Vazirinejad R, Ahmadi Z, Arababadi MK, Hassanshahi G, Kennedy D. The biological functions, structure and sources of CXCL10 and its outstanding part in the pathophysiology of multiple sclerosis. *Neuroimmunomodulation.* (2014) 21:322–30. doi: 10.1159/000357780
64. Sakai M, Ogawa K, Shiozaki A, Yoneda S, Sasaki Y, Nagata K, et al. Serum granulysin is a marker for Th1 type immunity in pre-eclampsia. *Clin Exp Immunol.* (2004) 136:114–9. doi: 10.1111/j.1365-2249.2004.02414.x
65. Zeilhofer HU, Schorr W. Role of interleukin-8 in neutrophil signaling. *Curr Opin Hematol.* (2000) 7:178–82. doi: 10.1097/00062752-200005000-00009
66. Lawlor KE, Campbell IK, Metcalf D, O'Donnell K, Van Nieuwenhuijze A, Roberts AW, et al. Critical role for granulocyte colony stimulating factor in inflammatory arthritis. *Proc Natl Acad Sci USA.* (2004) 101:11398–403. doi: 10.1073/pnas.0404328101
67. Martins A, Han J, Kim SO. The multifaceted effects of granulocyte colony-stimulating factor in immunomodulation and potential roles in intestinal immune homeostasis. *IUBMB Life.* (2010) 62:611–7. doi: 10.1002/iub.361
68. Bordon Y. Immune responses: IL-7 goes antiviral. *Nat Rev Immunol.* (2011) 11:158–9. doi: 10.1038/nri2949
69. Hoyer KK, Dooms H, Barron L, Abbas AK. Interleukin-2 in the development and control of inflammatory disease. *Immunol Rev.* (2008) 226:19–28. doi: 10.1111/j.1600-065X.2008.00697.x
70. Chinen T, Kannan AK, Levine AG, Fan X, Klein U, Zheng Y, et al. An essential role for the IL-2 receptor in T reg cell function. *Nat Immunol.* (2016) 17:1322–33. doi: 10.1038/ni.3540
71. Luzina IG, Keegan AD, Heller NM, Rook GAW, Atamas SP. Regulation of inflammation by interleukin - 4: a review of "alternatives." (2012) *J Leukoc Biol.* 92:753–64. doi: 10.1189/jlb.0412214
72. Iyer SS, Cheng G. Role of interleukin 10 transcriptional regulation in inflammation and autoimmune disease. *Crit Rev Immunol.* (2012) 32:23–63. doi: 10.1615/CritRevImmunol.v32.i1.30
73. Merad M, Martin JC. Pathological inflammation in patients with COVID-19: a key role for monocytes and macrophages. *Nat Rev Immunol.* (2020) 20:355–62. doi: 10.1038/s41577-020-0353-y
74. Aguilera ER, Lenz LL. Inflammation as a modulator of host susceptibility to pulmonary influenza, pneumococcal, and co-infections. *Front Immunol.* (2020) 11:105. doi: 10.3389/fimmu.2020.00105

75. Chan JFW, Lau SKP, To KKW, Cheng VCC, Woo PCY, Yue KY. Middle east respiratory syndrome coronavirus: another zoonotic betacoronavirus causing SARS-like disease. *Clin Microbiol Rev.* (2015) 28:465–522. doi: 10.1128/CMR.00102-14
76. Sato K, Misawa N, Takeuchi JS, Kobayashi T, Izumi T, Aso H, et al. Experimental adaptive evolution of SIVcpz to pandemic HIV-1 using a humanized mouse model. *J Virol.* (2017) 92:JVI.01905-17. doi: 10.1128/JVI.01905-17
77. Yi Y, Lagniton PNP, Ye S, Li E, Xu R-H. COVID-19: what has been learned and to be learned about the novel coronavirus disease. *Int J Biol Sci.*(2020) 16:1753–66. doi: 10.7150/ijbs.45134
78. Lu R, Zhao X, Li J, Niu P, Yang B, Wu H, et al. Genomic characterisation and epidemiology of 2019 novel coronavirus: implications for virus origins and receptor binding. *Lancet.* (2020) 395:565–74. doi: 10.1016/S0140-6736(20)30251-8
79. Hoffmann M, Kleine-Weber H, Krüger N, Müller M, Drosten C, Pöhlmann S. The novel coronavirus 2019 (2019-nCoV) uses the SARS-coronavirus receptor ACE2 and the cellular protease TMPRSS2 for entry into target cells. *bioRxiv*. (2020). doi: 10.1101/2020.01.31.929042
80. Hamming I, Timens W, Bulthuis MLC, Lely AT, Navis GJ, van Goor H. Tissue distribution of ACE2 protein, the functional receptor for SARS coronavirus. A first step in understanding SARS pathogenesis. *J Pathol.* (2004) 203:631–7. doi: 10.1002/path.1570
81. Tikellis C, Thomas MC. Angiotensin-converting enzyme 2 (ACE2) is a key modulator of the renin angiotensin system in health and disease. 2012. *Int J Peptides*. (2012) 20:62–8. doi: 10.1155/2012/256294
82. Al Heialy S, Hachim MY, Senok A, Abou Tayoun A, Hamoudi R, Alsheikh-Ali A, et al. Regulation of angiotensin converting enzyme 2 (ACE2) in obesity: implications for COVID-19. *bioRxiv*. (2020). doi: 10.1101/2020.04.17.046938
83. Liu PP, Blet A, Smyth D, Li H. The science underlying COVID-19: implications for the cardiovascular system. *Circulation.* (2020) 142:68–78. doi: 10.1161/CIRCULATIONAHA.120.047549
84. Xu Z, Shi L, Wang Y, Zhang J, Huang L, Zhang C, et al. Pathological findings of COVID-19 associated with acute respiratory distress syndrome. *Lancet Respir Med.* (2020) 8:420–2. doi: 10.1016/S2213-2600(20)30076-X
85. Gui M, Song W, Zhou H, Xu J, Chen S, Xiang Y, et al. Cryo-electron microscopy structures of the SARS-CoV spike glycoprotein reveal a prerequisite conformational state for receptor binding. *Cell Res.* (2017) 27:119–29. doi: 10.1038/cr.2016.152
86. Wrapp D, Wang N, Corbett KS, Goldsmith JA, Hsieh CL, Abiona O, et al. Cryo-EM structure of the 2019-nCoV spike in the prefusion conformation. *Science.* (2020) 367:1260–3. doi: 10.1126/science.abb2507
87. Kuba K, Imai Y, Rao S, Gao H, Guo F, Guan B, et al. A crucial role of angiotensin converting enzyme 2 (ACE2) in SARS coronavirus-induced lung injury. *Nat Med.* (2005) 11:875–9. doi: 10.1038/nm1267
88. Casey K, Iteen A, Nicolini R, Auten J. COVID-19 pneumonia with hemoptysis: acute segmental pulmonary emboli associated with novel coronavirus infection. *Am J Emerg Med.* (2020) 38:1544.e1–e3. doi: 10.1016/j.ajem.2020.04.011
89. Imai Y, Kuba K, Rao S, Huan Y, Guo F, Guan B, et al. Angiotensin-converting enzyme 2 protects from severe acute lung failure. *Nature.* (2005) 436:112–6. doi: 10.1038/nature03712
90. Channappanavar R, Perlman S. Pathogenic human coronavirus infections: causes and consequences of cytokine storm and immunopathology. *Semin Immunopathol.* (2017) 39:529–39. doi: 10.1007/s00281-017-0629-x
91. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet.* (2020) 395:497–506. doi: 10.1016/S0140-6736(20)30183-5
92. Zhou P, Yang X Lou, Wang XG, Hu B, Zhang L, Zhang W, et al. A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature.* (2020) 579:270–3. doi: 10.1038/s41586-020-2012-7
93. Wang X, Xu W, Hu G, Xia S, Sun Z, Liu Z, et al. SARS-CoV-2 infects T lymphocytes through its spike protein-mediated membrane fusion. *Cell Mol Immunol.* (2020) 1–3. doi: 10.1038/s41423-020-0424-9
94. Urra JM, Cabrera CM, Porras L, Ródenas I. Selective CD8 cell reduction by SARS-CoV-2 is associated with a worse prognosis and systemic inflammation in COVID-19 patients. *Clin Immunol.* (2020) 217:108486. doi: 10.1016/j.clim.2020.108486
95. Zheng Y, Huang Z, Yin G, Zhang X, Ye W, Hu Z, et al. Study of the lymphocyte change between COVID-19 and non-COVID-19 pneumonia cases suggesting other factors besides uncontrolled inflammation contributed to multi-organ injury. *SSRN Electron J.* (2020) 22. doi: 10.2139/ssrn.3555267
96. Li H, Liu L, Zhang D, Xu J, Dai H, Tang N, et al. SARS-CoV-2 and viral sepsis: observations and hypotheses. *Lancet.* (2020) 395:1517–20. doi: 10.1016/S0140-6736(20)30920-X
97. Tufan A, Avanoglu Güler A, Matucci-Cerinic M. Covid-19, immune system response, hyperinflammation and repurposinantirheumatic drugs. *Turkish J Med Sci.* (2020) 50:620–32. doi: 10.3906/sag-2004-168
98. Wan S, Yi Q, Fan S, Lv J, Zhang X, Guo L, et al. Characteristics of lymphocyte subsets and cytokines in peripheral blood of 123 hospitalized patients with 2019 novel coronavirus pneumonia (NCP). *medRxiv.* (2020). doi: 10.1101/2020.02.10.20021832
99. Wu Z, McGoogan JM. Characteristics of and important lessons from the coronavirus disease 2019 (COVID-19) outbreak in china: summary of a report of 72314 cases from the chinese center for disease control and prevention. *JAMA*. (2020) 323:1239–42. doi: 10.1001/jama.2020.2648
100. Cena H, Chieppa M. Coronavirus disease (COVID-19–SARS-CoV-2) and nutrition: is infection in italy suggesting a connection? *Front Immunol.* (2020) 11:944. doi: 10.3389/fimmu.2020.00944
101. Chiappetta S, Sharma AM, Bottino V, Stier C. COVID-19 and the role of chronic inflammation in patients with obesity. *Int J Obes.* (2020) 44:1790–2. doi: 10.1038/s41366-020-0597-4
102. Tsalamandris S, Antonopoulos AS, Oikonomou E, Papamikroulis GA, Vogiatzi G, Papaioannou S, et al. The role of inflammation in diabetes: current concepts and future perspectives. *Eur Cardiol Rev.* (2019) 14:50–9. doi: 10.15420/ecr.2018.33.1
103. Katsiari CG, Bogdanos DP, Sakkas LI. Inflammation and cardiovascular disease. *World J Transl Med.* (2019) 8:1–8. doi: 10.5528/wjtm.v8.i1.1
104. Childs CE, Calder PC, Miles EA. Diet and immune function. *Nutrients.* (2019) 11:1933. doi: 10.3390/nu11081933
105. Forouhi NG, Krauss RM, Taubes G, Willett W. Dietary fat and cardiometabolic health: evidence, controversies, and consensus for guidance. *BMJ.* (2018) 361:k2139. doi: 10.1136/bmj.k2139
106. Yaqoob P, Calder PC. Fatty acids and immune function: new insights into mechanisms. *Br J Nutr.* (2007) 98(Suppl. 1):19. doi: 10.1017/S0007114507832995
107. Kumar NG, Contaifer D, Madurantakam P, Carbone S, Price ET, Van Tassell B, et al. Dietary bioactive fatty acids as modulators of immune function: implications on human health. *Nutrients.* (2019) 11:1–15. doi: 10.3390/nu11122974
108. Karacabey K. The effect of nutritional elements on the immune system. *J Obes Weight Loss Ther.* (2012) 2:152. doi: 10.4172/2165-7904.1000152
109. Wolfe BE, Dutton RJ. Fermented foods as experimentally tractable microbial ecosystems. *Cell.* (2015) 161:49–55. doi: 10.1016/j.cell.2015.02.034
110. Montel MC, Buchin S, Mallet A, Delbes-Paus C, Vuitton DA, Desmasures N, et al. Traditional cheeses: rich and diverse microbiota with associated benefits. *Int J Food Microbiol.* (2014) 177:136–54. doi: 10.1016/j.ijfoodmicro.2014.02.019
111. Cameleyre M, Lytra G, Tempere S, Barbe JC. 2-Methylbutyl acetate in wines: enantiomeric distribution and sensory impact on red wine fruity aroma. *Food Chem.* (2017) 237:364–71. doi: 10.1016/j.foodchem.2017.05.093
112. Pandey A, Srivastava S, Rai P, Duke M. Cheese whey to biohydrogen and useful organic acids: a non-pathogenic microbial treatment by *L. acidophilus*. *Sci Rep.* (2019) 9:8320. doi: 10.1038/s41598-019-42752-3
113. Li S, Li P, Feng F, Luo LX. Microbial diversity and their roles in the vinegar fermentation process. *Appl Microbiol Biotechnol.* (2015) 99:4997–5024. doi: 10.1007/s00253-015-6659-1
114. Vinolo MAR, Rodrigues HG, Nachbar RT, Curi R. Regulation of inflammation by short chain fatty acids. *Nutrients.* (2011) 3:858–76. doi: 10.3390/nu3100858

115. Ratajczak W, Rył A, Mizerski A, Walczakiewicz K, Sipak O, Laszczynska M. Immunomodulatory potential of gut microbiome-derived shortchain fatty acids (SCFAs). *Acta Biochim Pol.* (2019) 66:1–12. doi: 10.18388/abp.2018_2648
116. Kaisar MMM, Pelgrom LR, van der Ham AJ, Yazdanbakhsh M, Everts B. Butyrate conditions human dendritic cells to prime type 1 regulatory T cells via both histone deacetylase inhibition and G protein-coupled receptor 109A signaling. *Front Immunol.* (2017) 8:1429. doi: 10.3389/fimmu.2017.01429
117. Liu T, Li J, Liu Y, Xiao N, Suo H, Xie K, et al. Short-Chain fatty acids suppress lipopolysaccharide-Induced production of nitric oxide and proinflammatory cytokines through inhibition of NF-?B Pathway in RAW264.7 cells. *Inflammation.* (2012) 35:1676–84. doi: 10.1007/s10753-012-9484-z
118. Macrae K, Stretton C, Lipina C, Blachnio-Zabielska A, Baranowski M, Gorski J, et al. Defining the role of DAG, mitochondrial function, and lipid deposition in palmitate-induced proinflammatory signaling and its counter-modulation by palmitoleate. *J Lipid Res.* (2013) 54:2366–78. doi: 10.1194/jlr.M036996
119. de Souza CO, Vannice GK, Rosa Neto JC, Calder PC. Is palmitoleic acid a plausible nonpharmacological strategy to prevent or control chronic metabolic and inflammatory disorders? *Mol Nutr Food Res.* (2018) 62:19. doi: 10.1002/mnfr.201700504
120. Passos MEP, Alves HHO, Momesso CM, Faria FG, Murata G, Cury-Boaventura MF, et al. Differential effects of palmitoleic acid on human lymphocyte proliferation and function. *Lipids Health Dis.* (2016) 15:1–11. doi: 10.1186/s12944-016-0385-2
121. Mayer K, Kiessling A, Ott J, Schaefer MB, Hecker M, Henneke I, et al. Acute lung injury is reduced in fat-1 mice endogenously synthesizing n-3 fatty acids. *Am J Respir Crit Care Med.* (2009) 179:474–83. doi: 10.1164/rccm.200807-1064OC
122. Bilal S, Haworth O, Wu L, Weylandt KH, Levy BD, Kang JX. Fat-1 transgenic mice with elevated omega-3 fatty acids are protected from allergic airway responses. *Biochim Biophys Acta.* (2011) 1812:1164–9. doi: 10.1016/j.bbadis.2011.05.002
123. Akbar U, Yang M, Kurian D, Mohan C. Omega-3 fatty acids in rheumatic diseases a critical review. *J Clin Rheumatol.* (2017) 23:330–9. doi: 10.1097/RHU.0000000000000563
124. Kiecolt-Glaser JK, Belury MA, Andridge R, Malarkey WB, Hwang BS, Glaser R. Omega-3 supplementation lowers inflammation in healthy middle-aged and older adults: a randomized controlled trial. *Brain Behav Immun.* (2012) 26:988–95. doi: 10.1016/j.bbi.2012.05.011
125. Jones GJB, Roper RL. The effects of diets enriched in omega-3 polyunsaturated fatty acids on systemic vaccinia virus infection. *Sci Rep.* (2017) 7:15999. doi: 10.1038/s41598-017-16098-7
126. Morita M, Kuba K, Ichikawa A, Nakayama M, Katahira J. The lipid mediator protectin D1 inhibits influenza virus replication and improves severe influenza. (2013) 11:12–3. doi: 10.1016/j.cell.2013.02.027
127. Kiecolt-Glaser JK, Belury MA, Andridge R, Malarkey WB, Glaser R. Omega-3 supplementation lowers inflammation and anxiety in medical students: a randomized controlled trial. *Brain Behav Immun.* (2011) 25:1725–34. doi: 10.1016/j.bbi.2011.07.229
128. Chowdhury R, Warnakula S, Kunutsor S, Crowe F, Ward HA, Johnson L, et al. Association of dietary, circulating, and supplement fatty acids with coronary risk: a systematic review and meta-analysis. *Ann Intern Med.* (2014) 160:398–406. doi: 10.7326/M13-1788
129. Patterson E, Wall R, Fitzgerald GF, Ross RP, Stanton C. Health implications of high dietary omega-6 polyunsaturated fatty acids 2012. *J Nutr Metabol.* (2012) 2012:539426. doi: 10.1155/2012/539426
130. Sergeant S, Rahbar E, Chilton FH. Gamma-linolenic acid, dihommo-gamma linolenic, eicosanoids and inflammatory processes. *Eur J Pharmacol.* (2017) 6:77–86. doi: 10.1016/j.ejphar.2016.04.020
131. Kapoor R, Huang Y. Gamma linolenic acid: an antiinflammatory omega-6 fatty acid. *Curr Pharm Biotechnol.* (2006) 7:531–4. doi: 10.2174/138920106779116874
132. Yao Q, Liu J, Zhang Z, Li F, Zhang C, Lai B, et al. Peroxisome proliferator-activated receptor γ (PPARγ) induces the gene expression of integrin αvβ5 to promote macrophage M2 polarization. *J Biol Chem.* (2019) 293:16572–82. doi: 10.1074/jbc.RA118.003161
133. Ciavarella C, Motta I, Valente S, Pasquinelli G. Pharmacological (or synthetic) and nutritional agonists of PPAR-γ as candidates for cytokine storm modulation in covid-19 disease. *Molecules.* (2020) 25:1–15. doi: 10.3390/molecules25092076
134. Håversen L, Danielsson KN, Fogelstrand L, Wiklund O. Induction of proinflammatory cytokines by long-chain saturated fatty acids in human macrophages. *Atherosclerosis.* (2009) 202:382–93. doi: 10.1016/j.atherosclerosis.2008.05.033
135. Lee JY, Sohn KH, Rhee SH, Hwang D. Saturated fatty acids, but not unsaturated fatty acids, induce the expression of cyclooxygenase-2 mediated through toll-like receptor 4. *J Biol Chem.* (2001) 276:16683–9. doi: 10.1074/jbc.M011695200
136. Ajuwon KM, Spurlock ME. Palmitate activates the NF-κB transcription factor and induces IL-6 and TNFα expression in 3T3-L1 adipocytes. *J Nutr.* (2005) 135:1841–6. doi: 10.1093/jn/135.8.1841
137. Gupta S, Knight AG, Gupta S, Keller JN, Bruce-Keller AJ. Saturated long-chain fatty acids activate inflammatory signaling in astrocytes. *J Neurochem.* (2012) 120:1060–71. doi: 10.1111/j.1471-4159.2012.07660.x
138. Stentz FB, Kitabchi AE. Palmitic acid-induced activation of human T-lymphocytes and aortic endothelial cells with production of insulin receptors, reactive oxygen species, cytokines, and lipid peroxidation. *Biochem Biophys Res Commun.* (2006) 346:721–6. doi: 10.1016/j.bbrc.2006.05.159
139. Limsuwat N, Boonarkart C, Phakaratsakul S, Suptawiwat O, Auewarakul P. Influence of cellular lipid content on influenza a virus replication. *Arch Virol.* (2020) 165:1151–61. doi: 10.1007/s00705-020-04596-5
140. Wypych TP, Marsland BJ, Ubags NDJ. The impact of diet on immunity and respiratory diseases. *Ann Am Thorac Soc.* (2017) 14:S339–47. doi: 10.1513/AnnalsATS.201703-255AW
141. Milner JJ, Sheridan PA, Karlsson EA, Schultz-Cherry S, Shi Q, Beck MA. Diet-induced obese mice exhibit altered heterologous immunity during a secondary 2009 pandemic H1N1 infection. *J Immunol.* (2013) 191:2474–85. doi: 10.4049/jimmunol.1202429
142. Kim Y, Kim J, Kim D, Nam J, Shim S, Choi Y, et al. Diet-induced obesity dramatically reduces the efficacy of a 2009 pandemic H1N1 vaccine in a mouse model. *J Infect Dis.* (2012) 341:10–1. doi: 10.1093/infdis/jir731
143. Smith AG, Sheridan PA, Harp JB, Beck MA. Diet-Induced obese mice have increased mortality and altered immune responses when infected with influenza virus. *J Nutr.* (2007) 137:1236–43. doi: 10.1093/jn/137.5.1236
144. Easterbrook JD, Dunfee RL, Schwartzman LM, Jagger BW, Sandouk A, Kash JC, et al. Obese mice have increased morbidity and mortality compared to non-obese mice during infection with the 2009 pandemic H1N1 influenza virus. *Influenza Other Respir Viruses.* (2011) 5:418–25. doi: 10.1111/j.1750-2659.2011.00254.x
145. Biron CA. Role of early cytokines, including alpha and beta interferons (IFN-α/β), in innate and adaptive immune responses to viral infections. *Semin Immunol.* (1998) 10:383–90. doi: 10.1006/smim.1998.0138
146. Samuels JD. Obesity and severe COVID-19 disease: a strong association. *Obesity.* (2020) 28:1368–68. doi: 10.1002/oby.22866
147. Horne JR, Vohl MC. Biological plausibility for interactions between dietary fat, resveratrol, ACE2, and SARS-CoV illness severity. *Am J Physiol Endocrinol Metab.* (2020) 318:E830–3. doi: 10.1152/ajpendo.00150.2020
148. Clemente JC, Ursell LK, Parfrey LW, Knight R. The impact of the gut microbiota on human health: an integrative view. *Cell.* (2012) 148:1258–70. doi: 10.1016/j.cell.2012.01.035
149. Trottier MD, Naaz A, Li Y, Fraker PJ. Enhancement of hematopoiesis and lymphopoiesis in diet-induced obese mice. *Proc Natl Acad Sci USA.* (2012) 109:7622–9. doi: 10.1073/pnas.1205129109
150. National Institutes of Health. *Nutrient Recommendations: Dietary Reference Intakes (DRI).* NihGov (2017). Available online at: https://ods.od.nih.gov/Health_Information/Dietary_Reference_Intakes.aspx
151. Esser N, Legrand-Poels S, Piette J, Scheen AJ, Paquot N. Inflammation as a link between obesity, metabolic syndrome and type 2 diabetes. *Diabetes Res Clin Pract.* (2014) 105:141–50. doi: 10.1016/j.diabres.2014.04.006
152. Yau YHC, Potenza MN. Stress and eating behaviors. *Minerva Endocrinol.* (2013) 38:255–67.
153. Myles IA. Fast food fever: reviewing the impacts of the western diet on immunity. *Nutr J.* (2014) 13:61. doi: 10.1186/1475-2891-13-61

154. Goldberg EL, Molony RD, Kudo E, Sidorov S, Kong Y, Dixit VD, et al. Ketogenic diet activates protective γδ T cell responses against influenza virus infection. *Sci Immunol.* (2019) 4:aav2026. doi: 10.1126/sciimmunol.aav2026
155. Sackner-Bernstein J, Kanter D, Kaul S. Dietary intervention for overweight and obese adults: comparison of low- carbohydrate and low-fat diets. A meta- analysis. *PLoS ONE.* (2015) 10:e0139817. doi: 10.1371/journal.pone.0139817
156. Mansoor N, Vinknes KJ, Veierod MB, Retterstol K. Effects of low-carbohydrate diets v. low-fat diets on body weight and cardiovascular risk factors a meta-analysis of randomised controlled trials. *Br J Nutr.* (2016) 115:466–79. doi: 10.1017/S0007114515004699
157. Cabrera-Mulero A, Tinahones A, Bandera B, Moreno-Indias I, Macías-González M, Tinahones FJ. Keto microbiota: a powerful contributor to host disease recovery. *Rev Endocr Metab Disord.* (2019) 20:415–25. doi: 10.1007/s11154-019-09518-8
158. Hussain A, Bhowmik B, Moreira V. COVID-19 and diabetes : knowledge in progress. (2020) 162:108142. doi: 10.1016/j.diabres.2020.108142
159. Wang D, Hu B, Hu C, Zhu F, Liu X, Zhang J, et al. Clinical characteristics of 138 hospitalized patients with 2019 novel coronavirus-infected pneumonia in Wuhan, China. *J Am Med Assoc.* (2020) 323:1061–9. doi: 10.1001/jama.2020.1585
160. Chen N, Zhou M, Dong X, Qu J, Gong F, Han Y, et al. Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study. *Lancet.* (2020) 395:507–13. doi: 10.1016/S0140-6736(20)30211-7
161. Bolla AM, Caretto A, Laurenzi A, Scavini M, Piemonti L. Low-carb and ketogenic diets in type 1 and type 2 diabetes. *Nutrients.* (2019) 11:962. doi: 10.3390/nu11050962
162. Barazzoni R, Bischoff SC, Breda J, Wickramasinghe K, Krznaric Z, Nitzan D, et al. ESPEN expert statements and practical guidance for nutritional management of individuals with SARS-CoV-2 infection. *Clin Nutr.* (2020) 39:1631–8. doi: 10.1016/j.clnu.2020.03.022
163. Yoneda J, Andou A, Takehana K. Regulatory roles of amino acids in immune response. *Curr Rheumatol Rev.* (2009) 5:252–8. doi: 10.2174/157339709790192567
164. Li P, Yin YL, Li D, Kim WS, Wu G. Amino acids and immune function. *Br J Nutr.* (2007) 98:237–52. doi: 10.1017/S000711450769936X
165. Farhan Aslam M, Majeed S, Aslam S, Irfan JA. Vitamins: key role players in boosting up immune response-a mini review. *Vitam Miner.* (2017) 6:1–8. doi: 10.4172/2376-1318.1000153
166. Taylor AK, Cao W, Vora KP, De La Cruz J, Shieh WJ, Zaki SR, et al. Protein energy malnutrition decreases immunity and increases susceptibility to influenza infection in mice. *J Infect Dis.* (2013) 207:501–10. doi: 10.1093/infdis/jis527
167. Brugliera L, Spina A, Castellazzi P, Cimino P, Arcuri P, Negro A, et al. Nutritional management of COVID-19 patients in a rehabilitation unit. *Eur J Clin Nutr.* (2020) 74:860–3. doi: 10.1038/s41430-020-0664-x
168. Cintoni M, Rinninella E, Annetta MG, Mele MC. Nutritional management in hospital setting during SARS-CoV-2 pandemic: a real-life experience. *Eur J Clin Nutr.* (2020) 74:846–7. doi: 10.1038/s41430-020-0625-4
169. Caccialanza R, Laviano A, Lobascio F, Montagna E, Bruno R, Ludovisi S, et al. Early nutritional supplementation in non-critically ill patients hospitalized for the 2019 novel coronavirus disease (COVID-19): Rationale and feasibility of a shared pragmatic protocol. *Nutrition.* (2020) 7:110835. doi: 10.1016/j.nut.2020.110835
170. Mcgaha TL, Huang L, Lemos H, Metz R, Mautino M, Prendergast GC, et al. Amino acid catabolism: a pivotal regulator of innate and adaptive immunity. *Immunol Rev.* (2012) 249:135–57. doi: 10.1111/j.1600-065X.2012.01149.x
171. Grimble RF. The Effects of Sulfur Amino Acid Intake on Immune Function in Humans. *J Nutr.* (2006) 136:1660S—5S. doi: 10.1093/jn/136.6.1660S
172. Calder PC. Branched-Chain Amino Acids and Immunity. *J Nutr.* (2006) 136:288S—93S. doi: 10.1093/jn/136.1.288S
173. Kim SW, Mateo RD, Yin YL, Wu G. Functional amino acids and fatty acids for enhancing production performance of sows and piglets. *Asian Austral J Anim Sci.* (2007) 20:295–306. doi: 10.5713/ajas.2007.295
174. Cruzat V, Rogero MM, Keane KN, Curi R, Newsholme P. Glutamine: metabolism and immune function, supplementation and clinical translation. *Nutrients.* (2018) 10:1564. doi: 10.3390/nu10111564
175. Newsholme EA, Newsholme P, Curi R. The role of the citric acid in cells of the immune system and its importance in sepsis, trauma and burns. *Biochem Soc Symp.* (1987) 54:145–61.
176. Crawford J, Cohen HJ. The essential role of L-glutamine in lymphocyte differentiation *in vitro*. *J Cell Physiol.* (1985) 124:275–82. doi: 10.1002/jcp.1041240216
177. Spittler A, Winkler S, Gotzinger P, Oehler R, Willhelm M, Tempfer C, et al. Influence of glutamine on the phenotype and function of human monocytes. *Blood.* (1995) 86:1564–9. doi: 10.1182/blood.V86.4.1564.bloodjournal8641564
178. Barbul A, Dawson H. Arginine and immunity. *Diet Nutr Immun.* (2018) 137:199–216. doi: 10.1201/9781351071390-15
179. Geiger R, Rieckmann JC, Wolf T, Basso C, Feng Y, Fuhrer T, et al. L-Arginine Modulates T Cell Metabolism and Enhances Survival and Anti-tumor Activity. *Cell.* (2016) 167:829–42.e13. doi: 10.1016/j.cell.2016.09.031
180. Mora JR, Iwata M, Von Andrian UH. Vitamin effects on the immune system: Vitamins A and D take centre stage. *Nat Rev Immunol.* (2008) 8:685–98. doi: 10.1038/nri2378
181. Maggini S, Wintergerst ES, Beveridge S, Hornig DH. Selected vitamins and trace elements support immune function by strengthening epithelial barriers and cellular and humoral immune responses. *Br J Nutr.* (2007) 98(Suppl. 1):29–35. doi: 10.1017/S0007114507832971
182. Gombart AF, Pierre A, Maggini S. A review of micronutrients and the immune system–working in harmony to reduce the risk of infection. *Nutrients.* (2020) 12:236. doi: 10.3390/nu12010236
183. Carr AC, Maggini S. Vitamin C and immune function. *Nutrients.* (2017) 9:1211. doi: 10.3390/nu9111211
184. Hemilä H. Vitamin C and infections. *Nutrients.* (2017) 9:9–10. doi: 10.3390/nu9040339
185. Hunt C, Chakravorty NK, Annan G, Habibzadeh N, Schorah CJ. The clinical effects of vitamin C supplementation in elderly hospitalised patients with acute respiratory infections. *Int J Vitam Nutr Res.* (1994) 64:212–9.
186. Ran L, Zhao W, Wang J, Wang H, Zhao Y, Tseng Y, et al. Extra dose of vitamin C based on a daily supplementation shortens the common cold: a meta-analysis of 9 randomized controlled trials. *Biomed Res Int.* (2018) 2018(Ci):1–2. doi: 10.1155/2018/1837634
187. PDQ Integrative, Alternative, and Complementary Therapies Editorial Board. High-dose vitamin C (PDQ®): health professional version. *PDQ Cancer Inf Summ.* (2002).
188. Carr AC, Rosengrave PC, Bayer S, Chambers S, Mehrtens J, Shaw GM. Hypovitaminosis C and vitamin C deficiency in critically ill patients despite recommended enteral and parenteral intakes. *Crit Care.* (2017) 21:300. doi: 10.1186/s13054-017-1891-y
189. Tina Suksmasari BH. Multivitamin supplementation supports immune function and ameliorates conditions triggered by reduced air quality. *Vitam Miner.* (2015) 04. doi: 10.4172/2376-1318.1000128
190. Lin Z, Li W. The roles of Vitamin D and its analogs in inflammatory diseases. *Curr Top Med Chem.* (2016) 16:1242–61. doi: 10.2174/1568026615666150915111557
191. Zhang Y, Leung DYM, Richers BN, Liu Y, Remigio LK, Riches DW, et al. Vitamin D inhibits monocyte/macrophage proinflammatory cytokine production by targeting MAPK phosphatase-1. *J Immunol.* (2012) 188:2127–35. doi: 10.4049/jimmunol.1102412
192. Cannell JJ, Vieth R, Umhau JC, Holick MF, Grant WB, Madronich S, et al. Epidemic influenza and vitamin D. *Epidemiol Infect.* (2006) 134:1129–40. doi: 10.1017/S0950268806007175
193. Grant WB, Lahore H, McDonnell SL, Baggerly CA, French CB, Aliano JL, et al. Evidence that vitamin d supplementation could reduce risk of influenza and covid-19 infections and deaths. *Nutrients.* (2020) 12:1–19. doi: 10.3390/nu12040988
194. Raharusun P, Priambada S, Budiarti C, Agung E, Budi C. Patterns of COVID-19 Mortality and Vitamin D: an Indonesian Study. *SSRN Electron J.* (2020). doi: 10.2139/ssrn.3585561
195. Rhodes JM, Subramanian S. Editorial: low population mortality from COVID-19 in countries south of latitude 35 degrees north supports vitamin d as a factor determining severity. *Aliment Pharmacol Ther.* (2020) 51:1434–7. doi: 10.1111/apt.15777

196. Iddir M, Brito A, Dingeo G, Fernandez Del Campo S, Samouda H, La Frano M, et al. Strengthening the immune system and reducing inflammation and oxidative stress through diet and nutrition: considerations during the COVID-19 Crisis. *Nutrients.* (2020) 12:1562. doi: 10.3390/nu12061562
197. Sommer A, Vyas KS. A global clinical view on vitamin A and carotenoids. *Am J Clin Nutr.* (2012) 96. doi: 10.3945/ajcn.112.034868
198. Stephensen CB. Vitamin A, infection, and immune function. *Annu Rev Nutr.* (2001) 21:167–92. doi: 10.1146/annurev.nutr.21.1.167
199. Villamor E, Fawzi WW. Effects of vitamin A supplementation on immune responses and correlation with clinical outcomes. *Clin Microbiol Rev.* (2005) 18:446–64. doi: 10.1128/CMR.18.3.446-464.2005
200. Calder PC. Feeding the immune system. *Proc Nutr Soc.* (2013) 72:299–309. doi: 10.1017/S0029665113001286
201. Huang Z, Liu Y, Qi G, Brand D, Zheng S. Role of vitamin A in the immune system. *J Clin Med.* (2018) 7:258. doi: 10.3390/jcm7090258
202. Wishart K. Increased micronutrient requirements during physiologically demanding situations: review of the current evidence. *Vitam Miner.* (2017) 6:166. doi: 10.4172/2376-1318.1000166
203. Aukrust P, Müller F, Ueland T, Svardal AM, Berge RK, Frøland SS. Decreased vitamin A levels in common variable immunodeficiency: Vitamin A supplementation *in vivo* enhances immunoglobulin production and downregulates inflammatory responses. *Eur J Clin Invest.* (2000) 30:252–9. doi: 10.1046/j.1365-2362.2000.00619.x
204. Maggini S, Beveridge S, Sorbara PJP, Senatore G. Feeding the immune system: The role of micronutrients in restoring resistance to infections. *CAB Rev Perspect Agric Vet Sci Nutr Nat Resour.* (2008) 3. doi: 10.1079/PAVSNNR20083098
205. Timoneda J, Rodríguez-Fernández L, Zaragozá R, Marín MP, Cabezuelo MT, Torres L, et al. Vitamin A deficiency and the lung. *Nutrients.* (2018) 10:1132. doi: 10.3390/nu10091132
206. Imdad A, Mayo-Wilson E, Herzer K, Bhutta ZA. Vitamin A supplementation for preventing morbidity and mortality in children from six months to five years of age. *Cochrane Database Syst Rev.* (2017) 2017:2020. doi: 10.1002/14651858.CD008524.pub3
207. Pelicano L, Li F, Schindler C, Chelbi-Alix MK. Retinoic acid enhances the expression of interferon-induced proteins: evidence for multiple mechanisms of action. *Oncogene.* (1997) 15:2349–59. doi: 10.1038/sj.onc.1201410
208. Chang HK, Hou WS. Retinoic acid modulates interferon-γ production by hepatic natural killer T cells via phosphatase 2A and the extracellular signal-regulated kinase pathway. *J Interf Cytokine Res.* (2015) 35:200–12. doi: 10.1089/jir.2014.0098
209. Soye KJ, Trottier C, Richardson CD, Ward BJ, Miller WH. RIG-I is required for the inhibition of measles virus by retinoids. *PLoS ONE.* (2011) 6:1–2. doi: 10.1371/journal.pone.0022323
210. Pichlmair A, Bearing SRNA, Responses I-MA, Responses I-MA, Bearing SRNA, Responses A, et al. RIG-I-mediated antiviral responses to single stranded RNA. *Science.* (2006) 314:997–1001. doi: 10.1126/science.1132998
211. Chew BP, Park JS. Carotenoid action on the immune response. *J Nutr.* (2004) 134:257S–61S. doi: 10.1093/jn/134.1.257S
212. Naithani R, Huma L, Holland L, Shukla D, McCormick D, Mehta R, et al. Antiviral activity of phytochemicals: a comprehensive review. *Mini Rev Med Chem.* (2008) 8:1106–33. doi: 10.2174/138955708785909943
213. Huang J, Weinstein SJ, Yu K, Männistö S, Albanes D. Serum beta carotene and overall and cause-specific mortality: a prospective cohort study. *Circ Res.* (2018) 123:1339–49. doi: 10.1161/CIRCRESAHA.118.313409
214. Bates CJ, Hamer M, Mishra GD. Redox-modulatory vitamins and minerals that prospectively predict mortality in older british people: the national diet and nutrition survey of people aged 65 years and over. *Br J Nutr.* (2011) 105:123–32. doi: 10.1017/S0007114510003053
215. Vrolijk MF, Opperhuizen A, Jansen EHJM, Godschalk RW, Van Schooten FJ, Bast A, et al. The shifting perception on antioxidants: the case of vitamin E and β-carotene. *Redox Biol.* (2015) 4:272–8. doi: 10.1016/j.redox.2014.12.017
216. Meydani SN, Leka LS, Fine BC, Dallal GE, Keusch GT, Singh MF, et al. Vitamin E and respiratory tract infections in elderly nursing home residents: a randomized controlled trial. *J Am Med Assoc.* (2004) 292:828–36. doi: 10.1001/jama.292.7.828
217. Hemilä H. Vitamin E administration may decrease the incidence of pneumonia in elderly males. *Clin Interv Aging.* (2016) 11:1379–85. doi: 10.2147/CIA.S114515
218. Levy M, Thaiss CA, Elinav E. Metabolites: messengers between the microbiota and the immune system. *Genes Dev.* (2016) 30:1589–97. doi: 10.1101/gad.284091.116
219. Yoshii K, Hosomi K, Sawane K, Kunisawa J. Metabolism of dietary and microbial vitamin b family in the regulation of host immunity. *Front Nutr.* (2019) 6:48. doi: 10.3389/fnut.2019.00048
220. Troen AM, Mitchell B, Sorensen B, Wener MH, Johnston A, Wood B, et al. Unmetabolized folic acid in plasma is associated with reduced natural killer cell cytotoxicity among postmenopausal women. *J Nutr.* (2006) 136:189–94. doi: 10.1093/jn/136.1.189
221. Cheng CH, Chang SJ, Lee BJ, Lin KL, Huang YC. Vitamin B6 supplementation increases immune responses in critically ill patients. *Eur J Clin Nutr.* (2006) 60:1207–13. doi: 10.1038/sj.ejcn.1602439
222. Bunout D, Barrera G, Hirsch S, Gattas V, De La Maza MP, Haschke F, et al. Effects of a nutritional supplement on the immune response and cytokine production in free-living Chilean elderly. *J Parenter Enter Nutr.* (2004) 28:348–54. doi: 10.1177/0148607104028005348
223. Mahmood L. The metabolic processes of folic acid and Vitamin B12 deficiency. *J Heal Res Rev.* (2014) 1:5. doi: 10.4103/2394-2010.143318
224. Tamura J, Kubota K, Murakami H, Sawamura M, Matsushima T, Tamura T, et al. Immunomodulation by vitamin B12: augmentation of CD8+ T lymphocytes and natural killer (NK) cell activity in vitamin B12-deficient patients by methyl-B12 treatment. *Clin Exp Immunol.* (1999) 116:28–32. doi: 10.1046/j.1365-2249.1999.00870.x
225. Schramm M, Wiegmann K. Riboflavin (vitamin B2) deficiency impairs NADPH oxidase 2 (Nox2) priming and defense against. eur j immunol. (2013). 44:728–41. doi: 10.1002/eji.201343940
226. Singh N, Gurav A, Sivaprakasam S, Brady E, Padia R, Shi H, et al. Activation of Gpr109a, receptor for niacin and the commensal metabolite butyrate, suppresses colonic inflammation and carcinogenesis. *Immunity.* (2014) 40:128–39. doi: 10.1016/j.immuni.2013.12.007
227. Lipszyc PS, Cremaschi GA, Zubilete MZ, Bertolino MLA, Capani F, Genaro AM, et al. Niacin modulates pro-inflammatory cytokine secretion. A potential mechanism involved in its anti-atherosclerotic effect. *Open Cardiovasc Med J.* (2013) 7:90–8. doi: 10.2174/1874192401307010090
228. Kennedy DO. B vitamins and the brain: mechanisms, dose and efficacy—a review. *Nutrients.* (2016) 8:68. doi: 10.3390/nu8020068
229. Ikeda M, Nagashima T, Nakamura E, Kato R, Ohshita M, Hayashi M, et al. *In vivo* roles of fatty acid biosynthesis enzymes in biosynthesis of biotin and α-lipoic acid in *Corynebacterium glutamicum*. *Appl Environ Microbiol.* (2017) 83:17. doi: 10.1128/AEM.01322-17
230. Elahi A, Sabui S, Narasappa NN, Agrawal S, Lambrecht NW, Agrawal A, et al. Biotin deficiency induces th1- and th17-mediated proinflammatory responses in human CD4 + T Lymphocytes via activation of the mTOR signaling pathway. *J Immunol.* (2018) 200:2563–70. doi: 10.4049/jimmunol.1701200
231. Agrawal S, Agrawal A, Said HM. Biotin deficiency enhances the inflammatory response of human dendritic cells. *Am J Physiol Cell Physiol.* (2016) 311:C386–91. doi: 10.1152/ajpcell.00141.2016
232. Wessels I, Rolles B, Rink L. The potential impact of zinc supplementation on COVID-19 pathogenesis. *Front Immunol.* (2020). 11:1712. doi: 10.3389/fimmu.2020.01712
233. Yakoob MY, Theodoratou E, Jabeen A, Imdad A, Eisele TP, Ferguson J, et al. Preventive zinc supplementation in developing countries: impact on mortality and morbidity due to diarrhea, pneumonia and malaria. *BMC Public Health.* (2011) 11(Suppl. 3):23. doi: 10.1186/1471-2458-11-S3-S23
234. Brooks WA, Santosham M, Naheed A, Goswami D, Wahed MA, Diener-West M, et al. Effect of weekly zinc supplements on incidence of pneumonia and diarrhoea in children younger than 2 years in an urban, low-income population in Bangladesh: randomised controlled trial. *Lancet.* (2005) 366:999–1004. doi: 10.1016/S0140-6736(05)67109-7

235. Girodon F, Lombard M, Galan P, Brunet-Lecomte P, Monget AL, Arnaud J, et al. Effect of micronutrient supplementation on infection in institutionalized elderly subjects: A controlled trial. *Ann Nutr Metab.* (1997) 41:98–107. doi: 10.1159/000177984
236. Prasad AS, Beck FWJ, Bao B, Fitzgerald JT, Snell DC, Steinberg JD, et al. Zinc supplementation decreases incidence of infections in the elderly: effect of zinc on generation of cytokines and oxidative stress. *Am J Clin Nutr.* (2007) 85:837–44. doi: 10.1093/ajcn/85.3.837
237. Meydani SN, Barnett JB, Dallal GE, Fine BC, Jacques PF, Leka LS, et al. Serum zinc and pneumonia in nursing home elderly. *Am J Clin Nutr.* (2007) 86:1167–73. doi: 10.1093/ajcn/86.4.1167
238. Read SA, Obeid S, Ahlenstiel C, Ahlenstiel G. The role of zinc in antiviral immunity. *Adv Nutr.* (2019) 10:696–710. doi: 10.1093/advances/nmz013
239. Saigal P, Hanekom D. Does zinc improve symptoms of viral upper respiratory tract infection? *Evidence-Based Pract.* (2019) 23:37–9. doi: 10.1097/EBP.0000000000000489
240. Wessling-Resnick M. Crossing the iron gate: why and how transferrin receptors mediate viral entry. *Annu Rev Nutr.* (2018) 38:431–58. doi: 10.1146/annurev-nutr-082117-051749
241. Tam M, Gómez S, González-Gross M, Marcos A. Possible roles of magnesium on the immune system. *Eur J Clin Nutr.* (2003) 57:1193–7. doi: 10.1038/sj.ejcn.1601689
242. Guillin OM, Vindry C, Ohlmann T, Chavatte L. Selenium, selenoproteins and viral infection. *Nutrients.* (2019) 11. doi: 10.3390/nu11092101
243. Beck MA, Handy J, Levander OA. Host nutritional status: the neglected virulence factor. *Trends Microbiol.* (2004) 12:417–23. doi: 10.1016/j.tim.2004.07.007
244. Beck MA, Kolbeck PC, Rohr LH, Shi Q, Morris VC, Levander OA. Benign human enterovirus becomes virulent in selenium-deficient mice. *J Med Virol.* (1994) 43:166–70. doi: 10.1002/jmv.1890430213
245. Hoffmann PR, Berry MJ. The influence of selenium on immune responses. *Mol Nutr Food Res.* (2008) 52:1273–80. doi: 10.1002/mnfr.200700330
246. Chelkeba L, Ahmadi A, Abdollahi M, Najafi A, Ghadimi MH, Mosaed R, et al. The effect of parenteral selenium on outcomes of mechanically ventilated patients following sepsis: a prospective randomized clinical trial. *Ann Intensive Care.* (2015) 5. doi: 10.1186/s13613-015-0071-y
247. Biban BG, Lichiardopol C. Iodine deficiency, still a global problem? *Curr Heal Sci J.* (2017) 43:103–11.
248. Bilal MY, Dambaeva S, Kwak-Kim J, Gilman-Sachs A, Beaman KD. A role for iodide and thyroglobulin in modulating the function of human immune cells. *Front Immunol.* (2017) 8:1573. doi: 10.3389/fimmu.2017.01573
249. Venturi S, Venturi M. Iodine, thymus, and immunity. *Nutrition.* (2020) 25:977–9. doi: 10.1016/j.nut.2009.06.002
250. Raha S, Mallick R, Basak S, Duttaroy AK. Is copper beneficial for COVID-19 patients? *Med Hypotheses.* (2020) 142:109814. doi: 10.1016/j.mehy.2020.109814
251. Muñoz C, Rios E, Olivos J, Brunser O, Olivares M. Iron, copper and immunocompetence. *Br J Nutr.* (2007) 98:S24–8. doi: 10.1017/S0007114507833046
252. Bala S, Failla ML. Copper deficiency reversibly impairs DNA synthesis in activated T lymphocytes by limiting interleukin 2 activity. *Proc Natl Acad Sci USA.* (1992) 89:6794–7. doi: 10.1073/pnas.89.15.6794
253. Besold AN, Culbertson EM, Culotta VC. The Yin and Yang of copper during infection. *J Biol Inorganic Chem.* (2016) 21:137–44. doi: 10.1007/s00775-016-1335-1
254. Weiss G, Carver PL. Role of divalent metals in infectious disease susceptibility and outcome. *Clin Microbiol Infect.* (2018) 24:16–23. doi: 10.1016/j.cmi.2017.01.018
255. Li C, Li Y, Ding C. The role of copper homeostasis at the host-pathogen axis: From bacteria to fungi. *Int Mol Sci.* (2019) 20:175. doi: 10.3390/ijms20010175
256. Reid D. Probiotics. (2005). 51:1455–7. doi: 10.2174/1381612053382395
257. Annunziata G, Sanduzzi Zamparelli M, Santoro C, Ciampaglia R, Stornaiuolo M, Tenore GC, et al. May polyphenols have a role against coronavirus infection? An overview of *in vitro* evidence. *Front Med.* (2020) 7:240. doi: 10.3389/fmed.2020.00240
258. Mani JS, Johnson JB, Steel JC, Broszczak DA, Neilsen PM, Walsh KB, et al. Natural product-derived phytochemicals as potential agents against coronaviruses: a review. *Virus Res.* (2020) 284197989. doi: 10.1016/j.virusres.2020.197989
259. Weng JR, Lin CS, Lai HC, Lin YP, Wang CY, Tsai YC, et al. Antiviral activity of sambucus formosanaNakai ethanol extract and related phenolic acid constituents against human coronavirus NL63. *Virus Res.* (2019) 273:197767. doi: 10.1016/j.virusres.2019.197767
260. Marinella MA. Indomethacin and resveratrol as potential treatment adjuncts for SARS-CoV-2/COVID-19. *Int J Clin Pract.* (2020). doi: 10.1111/ijcp.13535. [Epub ahead of print].
261. Zhang H, Chen Y, Chen Y, Ji S, Jia P, Li Y, et al. Comparison of the protective effects of resveratrol and pterostilbene against intestinal damage and redox imbalance in weanling piglets. *J Anim Sci Biotechnol.* (2020) 11:52. doi: 10.1186/s40104-020-00460-3
262. McCreary MR. *Randomized Proof-of-Concept Trial to Evaluate the Safety and Explore the Effectiveness of Resveratrol for COVID-19* (2020). Available online at: https://ClinicalTrials.gov
263. Wu D, Lewis ED, Pae M, Meydani SN. Nutritional modulation of immune function: analysis of evidence, mechanisms, and clinical relevance. *Front Immunol.* (2019) 9:3160. doi: 10.3389/fimmu.2018.03160
264. Dwivedi M, Kumar P, Laddha NC, Kemp EH. Induction of regulatory T cells: a role for probiotics and prebiotics to suppress autoimmunity. *Autoimmun Rev.* (2016) 15:379–92. doi: 10.1016/j.autrev.2016.01.002
265. Nagpal R, Kumar A, Kumar M, Behare P V., Jain S, Yadav H. Probiotics, their health benefits and applications for developing healthier foods: a review. *FEMS Microbiol Lett [Internet].* (2012) 334:1–15. doi: 10.1111/j.1574-6968.2012.02593.x
266. Galland L. The gut microbiome and the brain. *J Med Food.* (2014) 17:1261–72. doi: 10.1089/jmf.2014.7000
267. Rosenfeld CS. Microbiome disturbances and autism spectrum disorders. *Drug Metab Dispos.* (2015) 43:1557–71. doi: 10.1124/dmd.115.063826
268. Wang HY, Qi LW, Wang CZ, Li P. Bioactivity enhancement of herbal supplements by intestinal microbiota focusing on ginsenosides. *Am J Chin Med.* (2011) 39:1103–15. doi: 10.1142/S0192415X11009433
269. Vientós-Plotts AI, Ericsson AC, Rindt H, Reinero CR. Oral probiotics alter healthy feline respiratory microbiota. *Front Microbiol.* (2017) 8:1287. doi: 10.3389/fmicb.2017.01287
270. Dhar D, Mohanty A. Gut microbiota and Covid-19- possible link and implications. *Virus Res.* (2020) 285:198018. doi: 10.1016/j.virusres.2020.198018
271. Koch S, Nusrat A. The Life and Death of Epithelia During Inflammation: Lessons Learned from the Gut. *Annu Rev Pathol Mech Dis.* (2012) 7:35–60. doi: 10.1146/annurev-pathol-011811-120905
272. Yang H ying, Liu S ling, Ibrahim SA, Zhao L, Jiang J li, Sun W feng, et al. Oral administration of live Bifidobacterium substrains isolated from healthy centenarians enhanced immune function in BALB/c mice. *Nutr Res.* (2009) 29:281–9. doi: 10.1016/j.nutres.2009.03.010
273. Lee Y, Lee TS. Enhancement in *ex vivo* phagocytic capacity of peritoneal leukocytes in mice by oral delivery of various lactic-acid-producing bacteria. *Curr Microbiol.* (2005) 50:24–7. doi: 10.1007/s00284-004-4377-5
274. Tsai YT, Cheng PC, Fan CK, Pan TM. Time-dependent persistence of enhanced immune response by a potential probiotic strain *Lactobacillus paracasei* subsp. *paracasei* NTU 101. *Int J Food Microbiol.* (2008) 128:219–25. doi: 10.1016/j.ijfoodmicro.2008.08.009
275. Gill HS, Rutherfurd KJ, Cross ML. Dietary probiotic supplementation enhances natural killer cell activity in the elderly: an investigation of age-related immunological changes. *J Clin Immunol.* (2001) 21:264–71.
276. Parra MD, Martínez de Morentin BE, Cobo JM, Mateos A, Martínez JA. Daily ingestion of fermented milk containing *Lactobacillus casei* DN114001

improves innate-defense capacity in healthy middle-aged people. *J Physiol Biochem.* (2004) 60:85–91. doi: 10.1007/BF03168444
277. Trasino SE. A role for retinoids in the treatment of covid-19. *Clin Exp Pharmacol Physiol.* (2020) 19. doi: 10.1111/1440-1681.13354
278. Fu YR, Yi ZJ, Pei JL, Guan S. Effects of Bifidobacterium bifidum on adaptive immune senescence in aging mice. *Microbiol Immunol.* (2010) 54:578−83.
279. Mañé J, Pedrosa E, Lorén V, Gassull MA, Espadaler J, Cuñé J, et al. A mixture of Lactobacillus plantarum CECT 7315 and CECT 7316 enhances systemic immunity in elderly subjects. A dose-response, double-blind, placebo-controlled, randomized pilot trial. *Nutr Hosp.* (2011) 26:228–35.
280. Guillemard E, Tondu F, Lacoin F, Schrezenmeir J. Consumption of a fermented dairy product containing the probiotic *Lactobacillus casei* DN-114001 reduces the duration of respiratory infections in the elderly in a randomised controlled trial. *Br J Nutr.* (2010) 103:58–68. doi: 10.1017/S0007114509991395
281. Makino S, Ikegami S, Kume A, Horiuchi H, Sasaki H, Orii N. Reducing the risk of infection in the elderly by dietary intake of yoghurt fermented with *Lactobacillus delbrueckii* ssp. *bulgaricus* OLL1073R-1. *Br J Nutr.* (2010) 104:998–1006. doi: 10.1017/S000711451000173X
282. Shokryazdan P, Faseleh Jahromi M, Navidshad B, Liang JB. Effects of prebiotics on immune system and cytokine expression. *Med Microbiol Immunol.* (2017) 206:1–9. doi: 10.1007/s00430-016-0481-y
283. Kleessen B, Sykura B, Zunft HJ, Blaut M. Effects of inulin and lactose on fecal microflora, microbial activity, and bowel habit in elderly constipated persons. *Am J Clin Nutr.* (1997) 65:1397–402. doi: 10.1093/ajcn/65.5.1397
284. Bouhnik Y, Achour L, Paineau D, Riottot M, Attar A, Bornet F. Four-week short chain fructo-oligosaccharides ingestion leads to increasing fecal bifidobacteria and cholesterol excretion in healthy elderly volunteers. *Nutr J.* (2007) 6:42. doi: 10.1186/1475-2891-6-42
285. Markowiak P, Slizewska K. Effects of probiotics, prebiotics, and synbiotics on human health. *Nutrients.* (2017) 9:1021. doi: 10.3390/nu9091021
286. Mishra SP, Wang S, Nagpal R, Miller B, Singh R, Taraphder S, et al. Probiotics and prebiotics for the amelioration of type 1 diabetes: present and future perspectives. *Microorganisms.* (2019) 7:67. doi: 10.3390/microorganisms7030067
287. Anand S, Mande SS. Diet, microbiota and gut-lung connection. *Front Microbiol.* (2018) 9:2147. doi: 10.3389/fmicb.2018.02147
288. Conte L, Maurizio Toraldo D. Targeting the gut – lung microbiota axis by means of a high-fibre diet and probiotics may have anti-inflammatory effects in COVID-19 infection. *Ther Adv Respir Dis.* (2020) 1–5. doi: 10.1177/1753466620937170
289. Hanif S, Ali SN, Hassanein M, Khunti K, Hanif W. Managing people with diabetes fasting for Ramadan during the COVID-19 pandemic: a South Asian health foundation update. *Diabetic Med.* (2020) 37:1094–102. doi: 10.1111/dme.14312
290. Mosaferchi S, Sharif-Paghaleh E, Mortezapour A, Heidarimoghadam R. The first Ramadan during COVID-19 pandemic: 1.8 billion Muslims should fast or not? *Metabolism.* (2020) 108:154243. doi: 10.1016/j.metabol.2020.154253
291. Tootee A, Larijani B. Ramadan fasting during Covid-19 pandemic. *J Diabetes Metab Disord.* (2020) 19:1–4. doi: 10.1007/s40200-020-00534-z
292. Develioglu ON, Kucur M, Ipek HD, Celebi S, Can G, Kulekci M. Effects of ramadan fasting on serum immunoglobulin g and m, and salivary immunoglobulin a concentrations. *J Int Med Res.* (2013) 41:463–72. doi: 10.1177/0300060513476424
293. Faris MAIE, Kacimi S, Al-Kurd RA, Fararjeh MA, Bustanji YK, Mohammad MK, et al. Intermittent fasting during Ramadan attenuates proinflammatory cytokines and immune cells in healthy subjects. *Nutr Res.* (2012) 32:947–55. doi: 10.1016/j.nutres.2012.06.021
294. Norouzy A, Salehi M, Philippou E, Arabi H, Shiva F, Mehrnoosh S, et al. Effect of fasting in Ramadan on body composition and nutritionalintake: a prospective study. *J Hum Nutr Diet.* (2013) 26(Suppl.1):97–104. doi: 10.1111/jhn.12042
295. Ünalacak M, Kara IH, Baltaci D, Erdem Ö, Bucaktepe PGE. Effects of ramadan fasting on biochemical and hematological parameters and cytokines in healthy and obese individuals. *Metab Syndr Relat Disord.* (2011) 9:157–61. doi: 10.1089/met.2010.0084
296. Aksungar FB, Topkaya AE, Akyildiz M. Interleukin-6, C-reactive protein and biochemical parameters during prolonged intermittent fasting. *Ann Nutr Metab.* (2007) 51:88–95. doi: 10.1159/000100954
297. Akrami Mohajeri F, Ahmadi Z, Hassanshahi G, Akrami Mohajeri E, Ravari A, Razi Ghalebi S. Dose Ramadan fasting affects inflammatory responses: evidences for modulatory roles of this unique nutritional status via chemokine network. *Iran J Basic Med Sci.* (2013) 16:1217–22.
298. Schett G, Sticherling M, Neurath MF. COVID-19: risk for cytokine targeting in chronic inflammatory diseases? *Nat Rev Immunol.* (2020) 20:271–2. doi: 10.1038/s41577-020-0312-7
299. Ibrahim WH, Habib HM, Jarrar AH, Al Baz SA. Effect of Ramadan fasting on markers of oxidative stress and serum biochemical markers of cellular damage in healthy subjects. *Ann Nutr Metab.* (2009) 53:175–81. doi: 10.1159/000172979
300. Camini FC, da Silva Caetano CC, Almeida LT, de Brito Magalhães CL. Implications of oxidative stress on viral pathogenesis. *Arch Virol.* (2017) 162:907–17. doi: 10.1007/s00705-016-3187-y
301. Nikam S V., Nikam PS, Chandrashekar MR, Kalsad ST, Jnaneshwara KB. Role of lipid peroxidation, glutathione and antioxidant enzymes in H1N1 influenza. *Biomed Res.* (2010) 21:457–60.
302. Stekovic S, Hofer SJ, Tripolt N, Aon MA, Royer P, Pein L, et al. Alternate day fasting improves physiological and molecular markers of aging in healthy, non-obese humans. *Cell Metab.* (2019) 30:462–76.e5. doi: 10.1016/j.cmet.2019.07.016
303. Choi IY, Lee C, Longo VD. Nutrition and fasting mimicking diets in the prevention and treatment of autoimmune diseases and immunosenescence. *Mol Cell Endocrinol.* (2017) 455:4–12. doi: 10.1016/j.mce.2017.01.042
304. Jensen ME, Gibson PG, Collins CE, Hilton JM, Wood LG. Diet-induced weight loss in obese children with asthma: A randomized controlled trial. *Clin Exp Allergy.* (2013) 43:775–84. doi: 10.1111/cea.12115
305. Johnson JB, Summer W, Cutler RG, Martin B, Hyun DH, Dixit VD, et al. Alternate day calorie restriction improves clinical findings and reduces markers of oxidative stress and inflammation in overweight adults with moderate asthma. *Free Radic Biol Med.* (2007) 42:665–74. doi: 10.1016/j.freeradbiomed.2006.12.005
306. Adawi M, Watad A, Brown S, Aazza K, Aazza H, Zouhir M, et al. Ramadan fasting exerts immunomodulatory effects: Insights from a systematic review. *Front Immunol.* (2017) 8:1144. doi: 10.3389/fimmu.2017.01144
307. Palomares RA, Hurley DJ, Bittar JHJ, Saliki JT, Woolums AR, Moliere F, et al. Effects of injectable trace minerals on humoral and cell-mediated immune responses to Bovine viral diarrhea virus, Bovine herpes virus 1 and Bovine respiratory syncytial virus following administration of a modified-live virus vaccine in dairy calves. *Vet Immunol Immunopathol.* (2016) 178:88–98. doi: 10.1016/j.vetimm.2016.07.003

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Medicine

SYSTEMATIC REVIEW
published: 28 August 2020
doi: 10.3389/fmed.2020.00562

Incidence and Persistence of Viral Shedding in COVID-19 Post-acute Patients With Negativized Pharyngeal Swab: A Systematic Review

Giovanni Morone[1†], Angela Palomba[2†], Marco Iosa[1], Teodorico Caporaso[3], Domenico De Angelis[1], Vincenzo Venturiero[1], Anna Savo[1], Paola Coiro[1], Dario Carbone[4], Francesca Gimigliano[5], Giovanni Iolascon[2] and Stefano Paolucci[1]*

[1] Santa Lucia Foundation, Rome, Italy, [2] Multidisciplinary Department of Medicine for Surgery and Orthodontics, University of Campania Luigi Vanvitelli, Naples, Italy, [3] Department of Industrial Engineering, University of Naples Federico II, Naples, Italy, [4] Department of Anesthesia and Intensive Care Therapy, Military General Hospital, Rome, Italy, [5] Department of Mental and Physical Health and Preventive Medicine, University of Campania Luigi Vanvitelli, Naples, Italy

After the global spread of a severe acute respiratory syndrome caused by a coronavirus (SARS-CoV-2), factors that influence viral diffusion have gained great attention. Human-to-human transmission mainly occurs through droplets, but viral RNA clearance in different biological fluids in coronavirus disease 2019 (COVID-19) remains unclear. We aimed to correlate the presence and the relevant temporal patterns of SARS-CoV-2 viral RNA in biological specimens (stool, urine, blood, and tears) of the transmission with clinical/epidemiological features in patients with COVID-19. We focused on the time window between the positivity of reverse transcriptase-polymerase chain reaction (RT-PCR) tests from different specimens. We used the Mantel–Cox log rank test to verify the differences in terms of viral shedding duration, while we employed the Mann–Whitney *U*-test for subgroup analysis. This review protocol was registered with PROSPERO number: CRD42020183629. We identified 147 studies; we included 55 (1,348 patients) for epidemiological analysis, of which we included 37 (364 patients) for statistical analysis. The most frequently used specimens other than respiratory tract swabs were stool samples (or anal/rectal swabs), with a positivity rate of 48.8%, followed by urine samples, with a positivity rate of 16.4%; blood samples showed a positivity rate of 17.5%. We found that fecal positivity duration (median 19 days) was significantly ($p < 0.001$) longer than respiratory tract positivity (median 14 days). Limited data are available about the other specimens. In conclusion, medical and social communities must pay close attention to negativization criteria for COVID-19, because patients could have longer alternative viral shedding.

Keywords: viral shedding, COVID-19, SARS-CoV-2, stool, post-acute phase

OPEN ACCESS

Edited by:
Zhongheng Zhang,
Sir Run Run Shaw Hospital, China

Reviewed by:
Yanfei Shen,
Zhejiang Hospital, China
Jing Yuan,
Children's Hospital of Capital Institute of Pediatrics, China

***Correspondence:**
Marco Iosa
m.iosa@hsantalucia.it

† These authors have contributed equally to this work

Specialty section:
This article was submitted to Infectious Diseases – Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: *03 June 2020*
Accepted: *10 August 2020*
Published: *28 August 2020*

Citation:
Morone G, Palomba A, Iosa M, Caporaso T, De Angelis D, Venturiero V, Savo A, Coiro P, Carbone D, Gimigliano F, Iolascon G and Paolucci S (2020) Incidence and Persistence of Viral Shedding in COVID-19 Post-acute Patients With Negativized Pharyngeal Swab: A Systematic Review. Front. Med. 7:562. doi: 10.3389/fmed.2020.00562

INTRODUCTION

At the end of the 2019, a novel coronavirus was isolated from patients with pneumonia in Hubei province, China; it was named the 2019 novel coronavirus (2019-nCoV), and the related severe acute respiratory syndrome was referred to as SARS-CoV-2 (1). On January 30, 2020, the World Health Organization (WHO) announced that the new emerging coronavirus pneumonia epidemic constituted a public health emergency of international concern (2). On March 11, 2020, due to the exponential increase in the number of reported cases and the high number of deaths (3), WHO's General Director announced that the novel coronavirus disease (COVID-19) may be defined as a pandemic.

The main sources of infection are SARS-CoV-2-infected patients, who produce a large quantity of the virus in the upper respiratory tract during a prodromal period and clinical manifestations. However, many factors play a crucial role in augmenting diffusion, such as the presence of asymptomatic carriers, the incubation period of the disease (usually ranging from 1 to 14 days, and even up to 24 days), and the mild clinical symptoms during the first disease period, with infected subjects still having an active life (4, 5).

Our understanding of SARS-CoV-2 human-to-human transmission is still evolving; currently, we know that it mainly occurs through air droplets. However, feces may be another potential route of transmission (6). Nosocomial transmission is a severe problem, given the susceptible condition of inpatients, so any action should be taken to minimize the risk of transmission. Notably, there is no indication regarding the danger of biological fluids from a patient with a negative pharyngeal swab. This could become a major problem if he or she is admitted to a post-acute hospital ward or to any sanitary structure with lower healthcare assistance or when he or she is discharged into the community, as demonstrated by a recent review on gastrointestinal symptoms (7). Subjects with positive viral RNA excretion need to be isolated; however, the persistence and clearance of viral RNA in different biological fluids remains unclear. Thus, as the clearance of viral RNA from patients' stool is delayed compared with that from oropharyngeal swabs, it is important to detect the viral RNA in feces during the convalescence phase to provide guidance to patients about contact limitations and even to manage drug administration (i.e., avoiding immunosuppressant drugs such as glucocorticoids).

In this context, our study, inspired by the needs expressed by physicians in post-acute settings, aimed to systematically review the existing data on novel coronavirus viral shedding. We reviewed, referring to the recommended diagnostic criteria: (i) the incidence of viral RNA in biological specimens (urine, stool, blood, and tears); (ii) the persistence of viral shedding and the correlation between the presence of viral RNA in the respiratory tract and in feces; and (iii) the correlation between persistent viral shedding in the post-acute phase with disease severity.

METHODS

Search Strategy and Selection Criteria

For our systematic review and meta-analysis, we followed PRISMA guidelines (8). We searched for data on confirmed COVID-19 patients' viral shedding reported in any kind of study (case report/series, cohort studies, case-control studies, or randomized control trials) with available data in English, published until May 5, 2020. Two authors (G.M. and A.P.) independently and synchronously searched PubMed, EMBASE, and Web of Science up to May 5, 2020, in order to identify all studies documenting modalities of SARS-CoV-2 viral shedding in patients with a confirmed diagnosis of COVID-19.

The search terms were "2019-nCoV," "SARS-CoV-2," "novel coronavirus," or "COVID-19" combined with "viral shedding" and/or "feces," "stool," "feces," "urine," "blood," or "tears." We found additional studies by carefully searching the reference lists of the identified works. Titles and abstracts were screened, and two authors (G.M. and A.P.) independently reviewed full-text papers. Exclusion criteria were studies not written in English, not reporting specimens other than respiratory tract swabs, duplicates, or not matching the inclusion criteria and/or the topic of the review (for this last criterion, in case of disagreement between the two above authors, an independent reviewer stepped in, namely D.D.).

We obtained data about the sites of studies, sample sizes, patient demographics, analyzed clinical samples, disease duration, and viral shedding duration through different routes. We then focused on the time window between the positivity of reverse transcriptase-polymerase chain reaction (RT-PCR) tests from different specimens. In particular, we considered the duration of sample positivity for SARS-CoV-2 from the onset of symptoms or, for asymptomatic patients, from the first positive result until the last available positive testing. We considered respiratory samples (throat swabs, nasopharyngeal swabs, oral swabs, sputum, or saliva) to be a hallmark of COVID-19 diagnosis, while we compared the other specimens' duration of positivity to the respiratory one. We collected specific data about single patients when available. When possible, we asked corresponding authors for missing data in order to collect wider information. When we could not obtain single patient data, we took pooled data.

Data Analysis

M.I. performed all statistical analyses using Statistical Package for Social Science (SPSS) 25.0. Continuous variables are expressed as median (interquartile range) or mean ($\pm$ standard deviation), according to their normality test results (verified through the Shapiro–Wilk test). In order to overcome the possible heterogeneity within and between studies, M.I. performed the analyses on a pooled database containing data from each patient enrolled in the studies that provided single subject data and not using aggregated measures. M.I. used the Mantel–Cox log rank test to verify the differences in terms of viral shedding duration, while M.I. used the Mann–Whitney U-test for subgroup analysis. Moreover, we assessed the quality of the selected studies using the Newcastle–Ottawa Scale (9). We registered our review on PROSPERO (registration number: CRD42020183629).

RESULTS

The results of our search are shown in the PRISMA flow-chart depicted in **Figure 1**. After removal of duplicates and

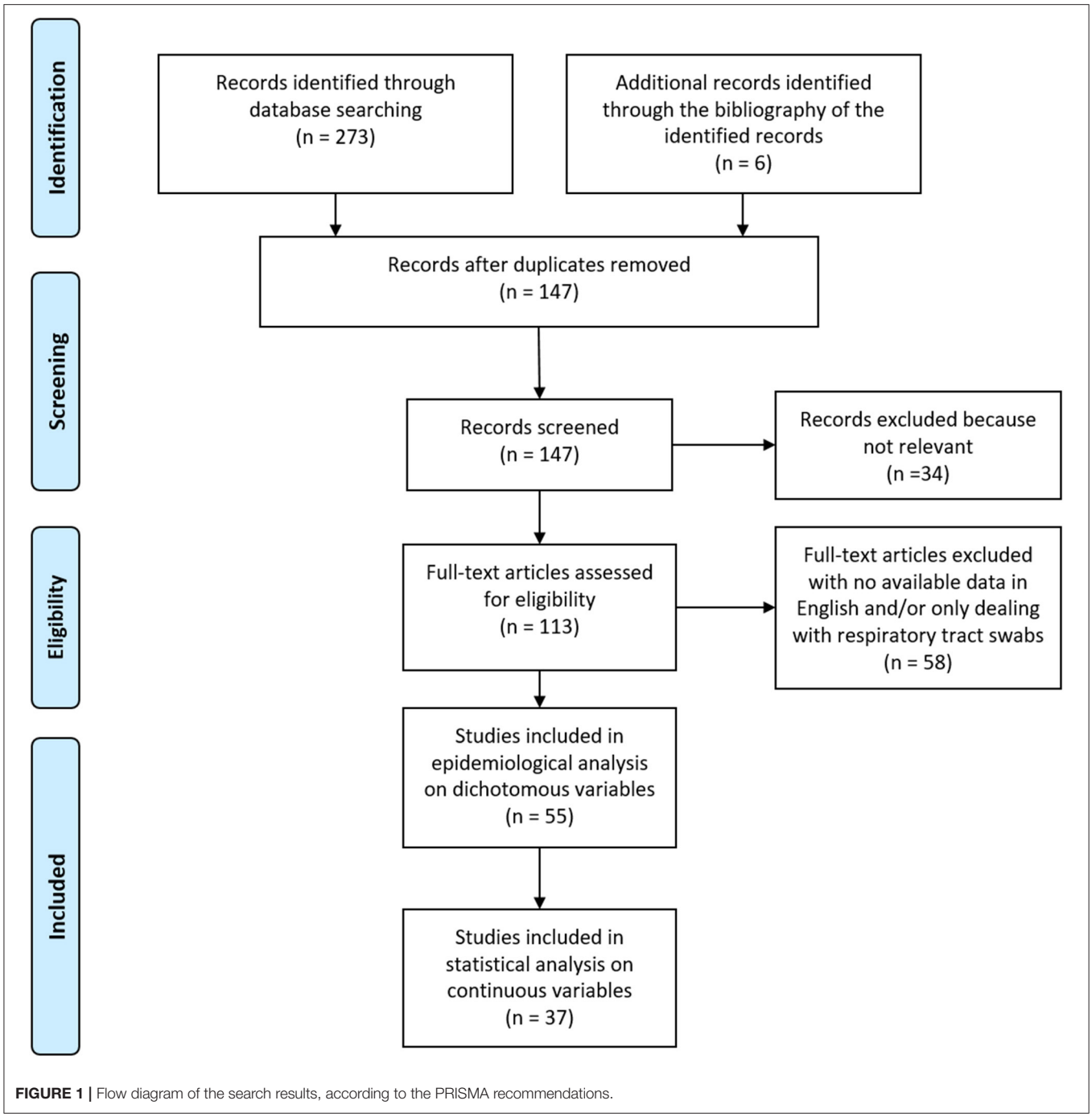


FIGURE 1 | Flow diagram of the search results, according to the PRISMA recommendations.

documents assessed as not eligible for our purposes, we found 113 papers. Of these, we included 55 studies in the present review for epidemiological analysis on group data and dichotomous variables; 37 of these reported continuous values and could be included in our quantitative analysis on single patients' data.

The detailed data of the 55 selected studies are available in **Table 1**. All the selected articles used RT-PCR for viral RNA detection (10–64). A few of them (32, 41, 42, 63) added viral cultures, viral isolation, or next generation sequencing (NGS). The total number of patients was 1,348 (1–132 for each article), with an age range from 17 days to 96 years. Of 1,219 patients for whom we found information about gender, 593 were female (48.6%). Most of the studies (78.2%) were conducted in China, while the others were from Asia (two from Korea, two from Singapore, one from Taiwan, and one from Lebanon), Europe (two from Italy, one from France, and one from Germany), and the United States of America (two). Almost all studies (52 out

TABLE 1 | Detailed information of the included studies (8–62).

	First author	Country	Sample	Method	Total patient number	Specimen positivity	Age range (years)	Sex	NOS
1	Wu	China	R, F	RT-PCR	74	41 F+	40–52	35 Fem 39 Mal	9
2	Zhang Y	China	R, F	RT-PCR	15	5 F+	37 (10–73)	7 Fem 8 Mal	8
3	Xu	China	R, F	RT-PCR	10	8 F+	0.2–15.7	4 Fem 6 Mal	8
4	Xing YH	China	R, F	RT-PCR	3	3 F+	1.5–6	1 Fem 2 Mal	8
5	Chen	China	R, F, U	RT-PCR	42	28 F+	51 (42.75–62)	24 Fem 18 Mal	9
6	Lo	China	R, F, U	RT-PCR	10	10 F+	54 (27–64)	7 Fem 3 Mal	9
7	Nicastri	Italy	R, F, U, O	RT-PCR	1	1 F+	29	1 Mal	7
8	Young	Singapore	R, F, Bl, U	RT-PCR	8	4 F+	n. a.	n. a.	7
9	Holshue	USA	R, F, Bl	RT-PCR	1	1 F+	35	1 Mal	7
10	Cai	China	R, F, Bl, U	RT-PCR	6	6 F+	0.3–10.9	4 Fem 2 Mal	8
11	Zhang JC	China	R, F	RT-PCR	14	5 F+	18–87	7 Fem 7 Mal	8
12	Zeng L	China	R, F	RT-PCR	1	1 F+	0.46	1 Mal	7
13	Yang Z	China	R, F	RT-PCR	3	3 F+	25–62	1 Fem 2 Mal	7
14	Xiao F	China	R, F	RT-PCR	73	39 F+	43 (0.83–7)	32 Fem 41 Mal	7
15	Zheng	China	R, F, Bl, U	RT-PCR	96	55 F+, 39 Bl+, 1 U+	55 (44.3–64.8)	38 Fem 58 Mal	8
16	Pan	China	R, F, U	RT-PCR	11	–	n. a.	n. a.	7
17	Cheng	Taiwan	R, F, U	RT-PCR	1	–	55	1 Fem	7
18	Kim	Korea	R, F, Bl, U	RT-PCR	2	–	35–55	1 Fem 1 Mal	8
19	Qian	China	R, F	RT-PCR	1	1 F+	47	1 Mal	7
20	Xing	China	R, F	RT-PCR	1	–	40	1 Mal	7
21	Tang	China	R, F	RT-PCR	1	1 F+	10	1 Mal	7
22	Tan	China	R, F, Bl	RT-PCR	4	3 F+	3.5–9	3 Fem 1 Mal	8
23	Mansour	Lebanon	R, F, U	RT-PCR/ cultures	1	–	1.41	1 Fem	7
24	Chen	China	R, F	RT-PCR	22	12 F+	2–64	8 Fem 14 Mal	9
25	Han	Korea	R, F, Bl, U	RT-PCR	2	1 F+ Bl+ U+ Sal+, 1 F+	55	2 Fem	7
26	Zhang T	China	R, F	RT-PCR	3	3 F+	6–9	3 Mal	8
27	Yuang	China	R, F	RT-PCR	6	6 F+	36–71	4 Fem 2 Mal	8
28	Liu	China	R, F	RT-PCR	4	4 F+	8–46	2 Fem 2 Mal	8
29	Li J	China	R, F, Bl, U, Vag, Mil	RT-PCR	13	3 F+	1–73	7 Fem 6 Mal	8
30	Jiang	China	R, F	RT-PCR	1	1 F+	8	1 Fem	7
31	Paoli	Italy	R, U, Sp	RT-PCR	1	–	31	1 Mal	7
32	Seah	Singapore	R, O	RT-PCR/viral isolation	17	–	20–75	6 Fem 11 Mal	7
33	Lescure	France	R, F, Bl, U, O	RT-PCR/viral isolation	5	2 F+, 1 Bl+	30–80	2 Fem 3 Mal	9
34	Wölfel	Germany	R, F, Bl, U	RT-PCR	16	8 F	35 (2–58)	4 Fem 12 Mal	7
35	Kujawski	USA	R, F, Bl, U	RT-PCR	10	6 F+, 1 F+ Bl+	53 (21–68)	3 Fem 7 Mal	9
36	Su	China	R, F	RT-PCR	4	4 F+	0.9–3.6	2 Fem 2 Mal	8
37	Sun	China	R, U	RT-PCR	1	1 U+	72	1 Mal	7
38	Cheung	China	R, F	RT-PCR	59	9 F+	22–96	32 Fem 27 Mal	6
39	Zhang W	China	R, F, Bl	RT-PCR	16	10 F+	n.a.	n.a.	7
40	Ling	China	R, F, U	RT-PCR	66	66 F+ (4/58 U+)	34–62	38 Fem 28 Mal	7
41	Lei	China	R, F	RT-PCR	7	4 F+	n.a.	n.a.	6
42	Wu	China	R, F, Bl	RT-PCR	132	36 F+, 4 Bl+	66.7 ± 9.1	60 F 72 M	7
43	Ma	China	R, F	RT-PCR	8	5 F+	0.9–39	6Fem 2 Mal	7
44	Fang	China	R, F, Bl, O	RT-PCR	32	23 Bl+; 5 O+	41 (34–54)	16 Fem 16 Mal	7

(Continued)

TABLE 1 | Continued

	First author	Country	Sample	Method	Total patient number	Specimen positivity	Age range (years)	Sex	NOS
45	Wei	China	R, F	RT-PCR	84	28 F+	37 (24–74)	56 Fem 28 Mal	7
46	Qian GQ	China	R, F	RT-PCR	91	2 F+	5–96	54 Fem 37 Mal	8
47	Peng	China	R, F, Bl, U	RT-PCR	7	1 F+, 1 F+ Bl+1 Bl+, 1 U+	27–49	4 Fem 3 Mal	7
48	Yun	China	R, F, Bl, O	RT-PCR	32	8 F+	50 (37–66)	17 Fem 15 Mal	7
49	Wang	China	R, U	RT-PCR	116	53 U+	54 (38-69)	49 Fem 67 Mal	7
50	Yu	China	R, Bl, U	RT-PCR/dd-PCR	76	4 Bl- 14 U-	40 (32–63)	38 Fem 38 Mal	8
51	Lin	China	R, F, Biop	RT-PCR	65	31 F+, 3/6 Biop+	n.a.	n.a.	7
52	Wang	China	R, F, Sew	RT-PCR	2	1 F+, Sew+	n.a.	n.a.	6
53	Xie	China	R, F, Bl, U	RT-PCR	9	8 F+	18–62	5 Fem 4 Mal	7
54	Huang J	China	R, Bl	RT-PCR/NGS	41	6 Bl+	49 (41–58)	11 Fem 30 Mal	8
55	Wang W	China	R, F, Bl	RT-PCR	20	6 F+, 2 Bl+	n.a.	n.a.	7

NOS, Newcastle—Ottawa Quality Assessment Scale; R, respiratory tract swabs; F, fecal samples; U, urine samples; Bl, blood; O, ocular samples; Vag, vaginal samples; Mil, Breast Milk; Biop, biopsies; Sew, sewage; +, number of positive samples; Fem, female; Mal, male; n. a., not available.

of 55) were assessed as high quality, showing Newcastle–Ottawa Scale scores $\geq$7 (9).

As shown in **Figure 2**, the most frequently used specimens other than respiratory tract swabs were stool samples (or anal/rectal swabs). Indeed, 50 articles examined fecal samples, with a positivity rate of 48.8% (490 out of 1,005 patients). Moreover, 22 articles examined urine samples, with a positivity rate of 16.4% (60 out of 366 patients), while blood samples showed in 20 articles a positivity rate of 17.5% (80 out of 456 patients). Finally, five articles considered ocular samples (tears or conjunctival swabs), with a positivity rate of 7.7% (5/65 patients). However, most of these studies did not report the duration data of each tested patient. One study (16) examined the semen of only one patient, with a negative result, while another study (38) looked for coronavirus RNA in the breast milk of a breastfeeding woman, also with a negative result. Another study (60) added the virus search on gastrointestinal tract biopsies (with three positive results out of six biopsies). Wang and colleagues (62) analyzed sewage and identified SARS-CoV-2 RNA.

From 37 studies (including 364 patients) reporting the duration of both R+ (respiratory tract swab positivity) and F+ (fecal sample positivity) for each patient (9–45), we pooled data for statistical analysis. Although these studies included 364 patients, R+ and F+ duration data were only available for 215 individuals, plus 11 patients for whom only the difference between F+ and R+ had been reported. The median R+ duration was 14 days [interquartile range (IQR) 12 days], whereas that of F+ was 19 days (IQR 14 days). The Shapiro–Wilk test highlighted that both R+ and F+ were not normally distributed ($p < 0.001$). For this reason, we used the Wilcoxon test to compare the lengths of positivity; there was a statistically significant difference ($p < 0.001$, $n = 215$). There was a significant correlation between the duration of R+ and F+ (Spearman correlation coefficient $R = 0.507$, $p < 0.001$). The Mantel–Cox log rank showed a statistically significant difference between F+ and R+ trends ($\chi^2 = 31.6$, $p < 0.001$; **Figure 3**). Of the 226 patients with both R+ and F+, 27 patients (11.9%) had the same duration for both routes of viral shedding, 55 (24.3%) had a longer R+ duration, and the remaining 144 (63.7%) showed a longer F+ duration.

Moreover, there were statistically significant differences between severe and not severe [as defined by the American Thoracic Society and Infectious Disease Society of America guidelines for community acquired pneumonia (65)] patients in terms of R+ duration ($p < 0.001$, Mann–Whitney U-test, $n = 309$), F+ duration ($p = 0.010$, $n = 184$), and their difference ($p < 0.001$, $n = 182$). Interestingly, for the most severe subjects, R+ and F+ durations were not statistically different from each other ($p = 0.496$, Wilcoxon test, $n = 69$), whereas for less severely affected patients, there was a statistically significant difference ($p < 0.001$, $n = 112$; **Figure 4**).

There were age data available for 105 subjects, 41 of whom were children (age < 18 years old), 38 of whom showed mild symptoms (according to the literature). For this reason, we compared the data of these 38 children with those of adults with mild symptoms for whom age data were available ($n = 48$). We did not find significant age-related differences in terms of R+ (median 8 days, IQR 9 days in children vs. median 10 days, IQR 11 days in adults; $p = 0.121$) or F+ (median 22 days, IQR 12 days in children vs. median 18 days, IQR 12 days in adults; $p = 0.058$). However, the difference between F+ and R+ was significantly longer in children than in adults (median 12 days, IQR 12 days in children vs. median 5 days, IQR 11 days in adults; $p = 0.001$).

Statistical analysis about the duration of other specimen positivity (blood, urine, and ocular samples) was not possible, due to the reduced sample size of available data. Indeed, single patient data about blood sample positivity were reported only in three studies (24, 34, 42), and urinary sample positivity duration was available for one single case study (34). There were no available data on other specimens for single patients.

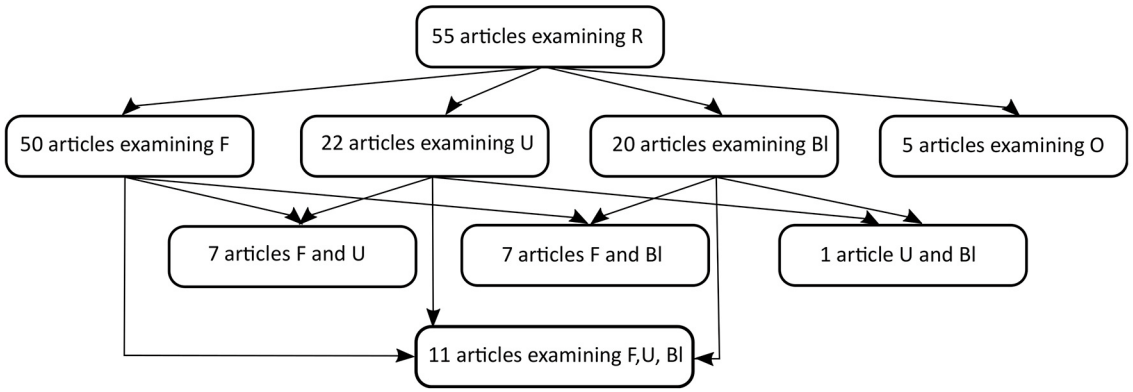


FIGURE 2 | Flow diagram of the specimens considered in the 55 selected articles (R, respiratory tract; F, feces; U, urine; Bl, Blood; O, ocular samples). We want to clarify that two of the F and Bl, one of the F, U, Bl and one of the F and U even consider ocular samples.

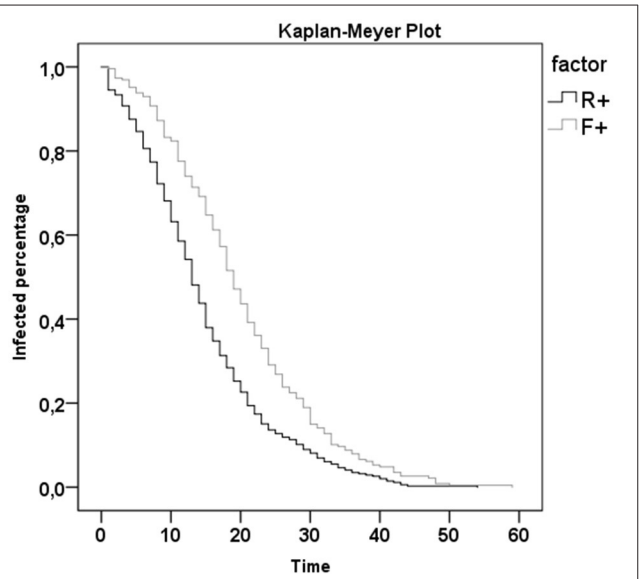


FIGURE 3 | Kaplan-Meyer Plot of R+ (respiratory tract swab positivity) and F+ (fecal sample positivity) duration.

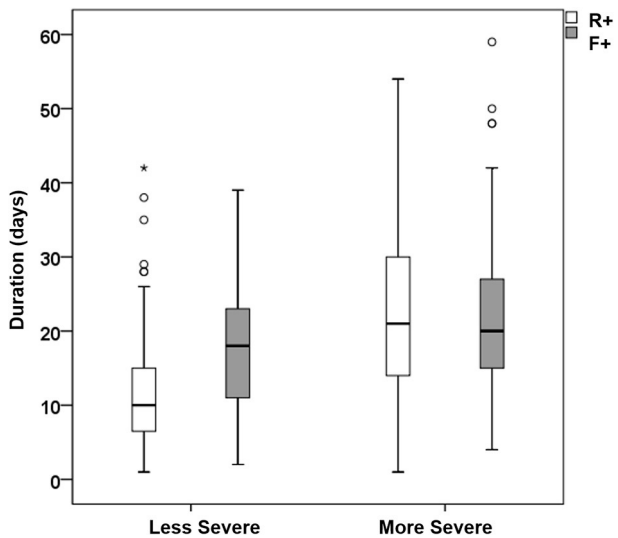


FIGURE 4 | Box-whiskers plot with median and quartile values for the duration of R+ (white boxes) and F+ (gray boxes) of less (left) and more (right) severe patients (circles and stars indicate patients out of the 95% confidence interval).

Finally, digestive symptoms were available for 42 patients, but all from the same study (14). For this reason, it was not possible to perform a meta-analysis on these symptoms.

DISCUSSION

The available data confirm the presence of viral RNA in several biological specimens (stool, urine, blood, and tears), but with very different positivity rates. Our results confirm concerns initially identified by Zhang and colleagues in their pioneering work (48). These concerns are related to modalities of dealing with people considered recovered after COVID-19 infection, without considering the persistent viral shedding in their biological specimens other than those collected in the respiratory tract. Not keeping them isolated or not taking the appropriate precautions could markedly increase the risk for virus spreading during the post-acute phase. Indeed, the present work confirms, on a wider basis than previous studies (364 patients), the significant prolonged viral shedding through feces. Although our aim was to also analyze other specimens, most of the analyzed studies only reported respiratory tract and fecal data. Our results revealed that the prolonged positivity of viral RNA excretion was statistically significant, particularly in patients with less severe disease, although digestive symptoms had only been anecdotally reported in previous review studies (14). This outcome may depend on the inclusion/exclusion search criteria of our review. Other reviews have focused on gastrointestinal symptoms and reported a higher prevalence in more severe patients (46, 65). Our findings suggest the importance of screening the viral positivity

of patients' stool even after negative results of their respiratory tract swabs. Therefore, prolonging the contact precautions both at home or in the post-acute environment for all post-COVID-19 patients seems to be advisable. The Kaplan–Meyer plot (**Figure 3**) would suggest prolonging the precautions for about 10 days. Moreover, as suggested by Yeo et al. (66), it is important to clarify the possibility of fecal-oral transmission for SARS-CoV-2, as already confirmed for other coronaviruses (67). In addition, a recent review by Cheung (47) investigated the correlation between fecal viral shedding duration and enteric symptoms. Finally, we analyzed the correlation between viral RNA excretion in feces and the disease severity. The longer duration of viral shedding in feces was statistically significant for less affected patients, and especially for children, a population in which the severity of COVID-19 was lower, as has been widely reported in literature.

The main limitation of this work is related to the fact that most of the studies detected viral RNA and not live viral shedding. So far, the exact correlation between RNA viral shedding and infectious viral shedding is not known, although live SAR-CoV-2 viruses have been isolated in different specimens including stool (68). We identified four other limitations: (1) despite our aim to analyze viral shedding in specimens other than respiratory swabs, most of the data were limited to feces; (2) specimens collected in different areas of the same body tract are considered a single type of sample; (3) we analyzed all data available in the publications about positivity rates and viral RNA shedding duration, but we must consider possible biases in the previous publications, for which only part of the data had been published by the authors (publication bias); and (4) the duration of infection might depend on the criteria related to the diagnosis of infection and to those for defining the negativization of a patient (with one or two consecutive negative tests) potentially related to different tracts (nasal or fecal swab tests) and different symptoms (respiratory or digestive).

CONCLUSIONS

In conclusion, on the basis of our results, medical and social communities must pay close attention to patients who present COVID-19 with mild or no symptoms, because our results suggest they could represent individuals with longer alternative viral shedding, even after a negativized pharyngeal swab. Therefore, appropriate management of the patient flow between an intensive care unit (ICU) and post-ICU departments (i.e., post-acute units) should be carefully considered by implementing risk management that is also related to alternative viral shedding.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

AUTHOR CONTRIBUTIONS

GM and AP performed the literature search and wrote the first draft of the report, with input from DC. TC assessed the quality of the selected studies. DD, VV, AS, PC, FG, GI, and SP reviewed the draft and expanded the clinical implications. MI performed the statistical analysis and had full access to all the data in the study. All authors contributed to the article and approved the submitted version.

FUNDING

This review has been supported by the Santa Lucia Foundation and the Italian Ministry of Healthcare (Current Research Line). The funders of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

REFERENCES

1. Zhu N, Zhang D, Wang, W, Li X, Yang B, Song J, et al. A novel coronavirus from patients with pneumonia in China, 2019. *N Engl J Med.* (2020) 382:727–33. doi: 10.1056/NEJMoa2001017
2. WHO. *WHO Director-General's Opening Remarks at the Media Briefing on COVID-19.* (2020). Available online at: https://www.who.int/dg/speeches/detail/who-director-general-s-opening-remarks-at-the-media-briefing-on-covid-19-11-march-2020 (accessed on May 10, 2020)
3. Iosa M, Paolucci S, Morone G. Covid-19: a dynamic analysis of fatality risk in Italy. *Front Med.* (2020) 7:185. doi: 10.3389/fmed.2020.00185
4. Zou L, Ruan F, Huang M, Liang L, Huang H, Hong Z, et al. SARS-CoV-2 viral load in upper respiratory specimens of infected patients. *N Engl J Med.* (2020) 382:1177–9. doi: 10.1056/NEJMc2001737
5. Rothe C, Schunk M, Sothmann P, Bretzel G, Froeschl G, Wallrauch C, et al. Transmission of 2019-nCoV infection from an asymptomatic contact in Germany. *N Engl J Med.* (2020) 382:970–1. doi: 10.1056/NEJMc2001468
6. Gu J, Han B, Wang, J COVID-19: gastrointestinal manifestations and potential fecal–oral transmission. *Gastroenterology.* (2020) 158:1518–19. doi: 10.1053/j.gastro.2020.02.054
7. Tu H, Tu S, Gao S, Shao A, Sheng J. The epidemiological and clinical features of COVID-19 and lessons from this global infectious public health event. *J Infect.* (2020) 81:1–9. doi: 10.1016/j.jinf.2020.04.011
8. Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. *PLoS Med.* (2009) 6:e1000097. doi: 10.1371/journal.pmed.1000097
9. Stang A. Critical evaluation of the Newcastle-Ottawa scale for the assessment of the quality of nonrandomized studies in meta-analyses. *Eur J Epidemiol.* (2010) 25:603–5. doi: 10.1007/s10654-010-9491-z
10. Wu Y, Guo C, Tang L, Hong Z, Zhou J, Dong X et al. Prolonged presence of SARS-CoV-2 viral RNA in faecal samples. *Lancet Gastroenterol Hepatol.* (2020) 5:434–5. doi: 10.1016/S2468-1253(20)30083-2
11. Yongchen Z, Shen H, Wang X, Shi X, Li Y, Yan J, et al. Different longitudinal patterns of nucleic acid and serology testing results based on disease severity of COVID-19 patients. *Emerg Microbes Infect.* (2020) 9:833–6. doi: 10.1080/22221751.2020.1756699
12. Xu Y, Li X, Zhu B, Liang H, Fang C, Gong Y, et al. Characteristics of pediatric SARS-CoV-2 infection and potential evidence for persistent fecal viral shedding. *Nat Med.* (2020) 26:502–5. doi: 10.1038/s41591-020-0817-4
13. Xing YH, Ni W, Wu Q, Li WJ, Li GJ, Wang WD, et al. Prolonged viral shedding in feces of pediatric patients with coronavirus disease 2019. *J Microbiol Immunol Infect.* (2020) 53:473–80. doi: 10.1016/j.jmii.2020.03.021
14. Chen Y, Chen L, Deng Q, Zhang G, Wu K, Ni L, et al. The presence of SARS-CoV-2 RNA in feces of COVID-19 patients. *J Med Virol.* (2020) 92:833–40. doi: 10.1002/jmv.25825

15. Lo IL, Lio CF, Cheong HH, Lei CI, Cheong TH, Zhong X, et al. Evaluation of SARS-CoV-2 RNA shedding in clinical specimens and clinical characteristics of 10 patients with COVID-19 in Macau. *Int J Biol Sci*. (2020) 16:1698–707. doi: 10.7150/ijbs.45357
16. Nicastri E, D'Abramo A, Faggioni G, De Santis R, Mariano A, Lepore L, et al. Coronavirus disease (COVID-19) in a paucisymptomatic patient: epidemiological and clinical challenge in settings with limited community transmission. *Euro Surveill*. (2020) 25:2000230. doi: 10.2807/1560-7917.ES.2020.25.11.2000230
17. Young BE, Ong SWX, Kalimuddin S, Low JG, Tan SY, Loh J, et al. Epidemiologic features and clinical course of patients infected with SARS-CoV-2 in Singapore. *JAMA*. (2020) 323:1488–94. doi: 10.1001/jama.2020.3204
18. Holshue ML, DeBolt C, Lindquist S, Lofy KH, Wiesman J, Bruce H, et al. First case of 2019 novel coronavirus in the United States. *N Engl J Med*. (2020) 382:929–36. doi: 10.1056/NEJMoa2001191
19. Cai J, Xu J, Lin D, Xu L, Qu Z, Zhang Y, et al. A case series of children with 2019 novel coronavirus infection: clinical and epidemiological features. *Clin Infect Dis*. (2020). doi: 10.1093/cid/ciaa198. [Epub ahead of print].
20. Zhang J, Wang S, Xue Y. Fecal specimen diagnosis 2019 novel coronavirus–infected pneumonia. *J Med Virol*. (2020) 92:680–2. doi: 10.1002/jmv.25742
21. Zeng LK, Tao XW, Yuan WH, Wang J, Liu X, Liu ZS. First case of neonate infected with novel coronavirus pneumonia in China. *Zhonghua Er Ke Za Zhi*. (2020) 58:E009. doi: 10.3760/cma.j.issn.0578-1310.2020.0009
22. Yang Z, Li G, Dai X, Liu G, Li G, Jie Y. Three cases of novel coronavirus pneumonia with viral nucleic acids still positive in stool after throat swab detection turned negative. *Chin J Dig*. (2020) 40:E002. doi: 10.3760/cma.j.issn.0254-1432.2020.0002
23. Xiao F, Tang M, Zheng X, Liu Y, Li X, Shan H. Evidence for gastrointestinal infection of SARS-CoV-2. *Gastroenterology*. (2020) 158:1831–3.e3. doi: 10.1053/j.gastro.2020.02.055
24. Zheng S, Fan J, Yu F, Feng B, Lou B, Zou Q, et al. Viral load dynamics and disease severity in patients infected with SARS-CoV-2 in Zhejiang province, China, January-March 2020: retrospective cohort study. *BMJ*. (2020) 369:m1443. doi: 10.1136/bmj.m1443
25. Pan Y, Zhang D, Yang P, Poon LL, Wang Q. Viral load of SARS-CoV-2 in clinical samples. *Lancet Infect Dis*. (2020) 20:411–2. doi: 10.1016/S1473-3099(20)30113-4
26. Cheng SC, Chang YC, Chiang YLF, Chien YC, Cheng M, Yang CH, et al. First case of Coronavirus Disease 2019 (COVID-19) pneumonia in Taiwan. *J Formos Med Assoc*. (2020) 119:747–51. doi: 10.1016/j.jfma.2020.02.007
27. Kim JY, Ko JH, Kim Y, Kim YJ, Kim JM, Chung YS, et al. Viral load kinetics of SARS-CoV-2 infection in first two patients in Korea. *J Korean Med Sci*. (2020) 35:e86. doi: 10.3346/jkms.2020.35.e86
28. Qian GQ, Chen XQ, Lv DF, Ma AHY, Wang LP, Yang NB, et al. Duration of SARS-CoV-2 viral shedding during COVID-19 infection. *Infect Dis*. (2020) 52:511–12. doi: 10.1080/23744235.2020.1748705
29. Xing Y, Mo P, Xiao Y, Zhang Y, Wang F. Post-discharge surveillance and positive virus detection in two medical staff recovered from coronavirus disease 2019 (COVID-19), China, January to February 2020. *Euro Surveill*. (2020) 25:2000191. doi: 10.2807/1560-7917.ES.2020.25.10.2000191
30. Tang A, Tong ZD, Wang HL, Dai YX, Li KF, Liu JN. Detection of novel coronavirus by RT-PCR in stool specimen from asymptomatic child, China. *Emerg Infect Dis*. (2020) 26:1337–9. doi: 10.3201/eid2606.20.0301
31. Tan YP, Tan BY, Pan J, Wu, J, Zeng SZ, Wei HY. Epidemiologic and clinical characteristics of 10 children with coronavirus disease 2019 in Changsha, China. *J Clin Virol*. (2020) 127:104353. doi: 10.1016/j.jcv.2020.104353
32. Mansour A, Atoui R, Kamal Kanso RM, Fares Y, Fares J. First case of an Infant with COVID-19 in the middle east. *Cureus*. (2020) 12:e7520. doi: 10.7759/cureus.7520
33. Chen C, Gao G, Xu Y, Pu L, Wang Q, Wang L, et al. SARS-CoV-2–positive sputum and feces after conversion of pharyngeal samples in patients with COVID-19. *Ann Intern Med*. (2020) 172:832–4. doi: 10.7326/M20-0991
34. Han MS, Seong MW, Heo EY, Park JH, Kim N, Shin S, et al. Sequential analysis of viral load in a neonate and her mother infected with SARS-CoV-2. *Clin Infect Dis*. (2020). doi: 10.1093/cid/ciaa447. [Epub ahead of print].
35. Zhang T, Cui X, Zhao X, Wang J, Zheng J, Zheng G, et al. Detectable SARS-CoV-2 viral RNA in feces of three children during recovery period of COVID-19 pneumonia. *J Med Virol*. (2020) 92:909–14. doi: 10.1002/jmv.25795
36. Wang N, Ou X. Caution should be exercised for the detection of SARS-CoV-2, especially in the elderly. *J Med Virol*. (2020). doi: 10.1002/jmv.25796. [Epub ahead of print].
37. Liu J, Xiao Y, Shen Y, Shi C, Chen Y, Shi P, et al. Detection of SARS-CoV-2 by RT-PCR in anal from patients who have recovered from coronavirus disease 2019. *J Med Virol*. (2020). doi: 10.1002/jmv.25875. [Epub ahead of print].
38. Li Y, Hu Y, Yu Y, Zhang X, Li B, Wu J, et al. Positive result of Sars-Cov-2 in faeces and sputum from discharged patient with COVID-19 in Yiwu, China. *J Med Virol*. (2020). doi: 10.1002/jmv.25905. [Epub ahead of print].
39. Jiang X, Luo M, Zou Z, Wang X, Chen C, Qiu, J. Asymptomatic SARS-CoV-2 infected case with viral detection positive in stool but negative in nasopharyngeal samples lasts for 42 days. *J Med Virol*. (2020). doi: 10.1002/jmv.25941. [Epub ahead of print].
40. Paoli D, Pallotti F, Colangelo S, Basilico F, Mazzuti L, Turriziani O, et al. Study of SARS-CoV-2 in semen and urine samples of a volunteer with positive naso-pharyngeal swab. *J Endocrinol Invest*. (2020). doi: 10.1007/s40618-020-01261-1. [Epub ahead of print].
41. Jun ISY, Anderson DE, Kang AEZ, Wang LF, Rao P, Young BE, et al. Assessing viral shedding and infectivity of tears in coronavirus disease 2019 (COVID-19) patients. *Ophthalmology*. (2020) 127:977–79. doi: 10.1016/j.ophtha.2020.03.026
42. Lescure FX, Bouadma L, Nguyen D, Parisey M, Wicky PH, Behillil S, et al. Clinical and virological data of the first cases of COVID-19 in Europe: a case series. *Lancet Infect Dis*. (2020) 20:697–706. doi: 10.1016/S1473-3099(20)30200-0
43. Wölfel R, Corman VM, Guggemos W, Seilmaier M, Zange S, Müller MA, et al. Virological assessment of hospitalized patients with COVID-2019. *Nature*. (2020) 581:465–69. doi: 10.1038/s41586-020-2196-x
44. Kujawski SA, Wong KK, Collins JP, Epstein L, Killerby ME, Midgley CM, et al. Clinical and virologic characteristics of the first 12 patients with coronavirus disease 2019 (COVID-19) in the United States. *Nat Med*. (2020) 26:861–68. doi: 10.1038/s41591-020-0877-5
45. Su L, Ma X, Yu H, Zhang Z, Bian P, Han Y, et al. The different clinical characteristics of corona virus disease cases between children and their families in China–the character of children with COVID-19. *Emerg Microbes Infect*. (2020) 9:707–13. doi: 10.1080/22221751.2020.1744483
46. Sun J, Zhu A, Li H, Zheng K, Zhuang Z, Chen Z, et al. Isolation of Infectious SARS-CoV-2 from Urine of a COVID-19 Patient. *Emerg Microbes Infect*. (2020) 9:991–93. doi: 10.1080/22221751.2020.1760144
47. Cheung KS, Hung IF, Chan PP, Lung KC, Tso E, Liu R, et al. Gastrointestinal manifestations of SARS-CoV-2 infection and virus load in fecal samples from the Hong Kong cohort and systematic review and meta-analysis. *Gastroenterology*. (2020). 159:81–95. doi: 10.1053/j.gastro.2020.03.065
48. Zhang W, Du RH, Li B, Zheng XS, Yang XL, Hu B, et al. Molecular and serological investigation of 2019-nCoV infected patients: implication of multiple shedding routes. *Emerg Microbes Infect*. (2020) 9:386–9. doi: 10.1080/22221751.2020.1729071
49. Ling Y, Xu SB, Lin YX, Tian D, Zhu ZQ, Dai FH, et al. Persistence and clearance of viral RNA in 2019 novel coronavirus disease rehabilitation patients. *Chin Med J*. (2020) 133:1039–43. doi: 10.1097/CM9.0000000000000774
50. Lei Z, Cao H, Jie Y, Huang Z, Guo X, Chen J, et al. A cross-sectional comparison of epidemiological and clinical features of patients with coronavirus disease (COVID-19) in Wuhan and outside Wuhan, China. *Travel Med Infect Dis*. (2020) 35:101664. doi: 10.1016/j.tmaid.2020.101664
51. Wu J, Liu J, Li S, Peng Z, Xiao Z, Wang X, et al. Detection and analysis of nucleic acid in various biological samples of COVID-19 patients. *Travel Med Infect Dis*. (2020). doi: 10.1016/j.tmaid.2020.101673. [Epub ahead of print].
52. Ma X, Su L, Zhang Y, Zhang X, Gai Z, Zhang Z. Do children need a longer time to shed SARS-CoV-2 in stool than adults? *J Microbiol Immunol Infect*. (2020) 53:373–76. doi: 10.1016/j.jmii.2020.03.010
53. Fang Z, Zhang Y, Hang C, Ai J, Li S, Zhang W. Comparisons of viral shedding time of SARS-CoV-2 of different samples in ICU and non-ICU patients. *J Infect*. (2020) 81:147–78. doi: 10.1016/j.jinf.2020.03.013
54. Wei XS, Wang X, Niu YR, Ye LL, Peng WB, Wang ZH, et al. Diarrhea is associated with prolonged symptoms and viral carriage in COVID-19. *Clin Gastroenterol Hepatol*. (2020) 18:1753–1759.e2. doi: 10.1016/j.cgh.2020.04.030

55. Qian GQ, Yang NB, Ding F, Ma AHY, Wang ZY, Shen YF, et al. Epidemiologic and clinical characteristics of 91 hospitalized patients with COVID-19 in Zhejiang, China: a retrospective, multi-centre case series. *QJM*. (2020) 113:474–81. doi: 10.1093/qjmed/hcaa089
56. Peng L, Liu J, Xu W, Luo Q, Deng K, Lin B, et al. SARS-CoV-2 can be detected in urine, blood, anal swabs, and oropharyngeal swabs specimens. *J Med Virol*. (2020). doi: 10.1002/jmv.25936. [Epub ahead of print].
57. Yun H, Sun Z. Wu J, Tang A, Hu M, Xiang Z. Laboratory data analysis of novel coronavirus (COVID-19) screening in 2510 patients. *Clin Chim Acta*. (2020) 507:94–7. doi: 10.1016/j.cca.2020.04.018
58. Wang L, Li X, Chen H, Yan S, Li D, Li Y, et al. Coronavirus disease 19 infection does not result in acute kidney injury: an analysis of 116 hospitalized patients from Wuhan, China. *Am J Nephrol*. (2020) 51:343–8. doi: 10.1159/000507471
59. Yu F, Yan L, Wang N, Yang S, Wang L, Tang Y, et al. Quantitative detection and viral load analysis of SARS-CoV-2 in infected patients. *Clin Infect Dis*. (2020) 71:793–98. doi: 10.1093/cid/ciaa345
60. Lin L, Jiang X, Zhang Z, Huang S, Zhang Z, Fang Z, et al. Gastrointestinal symptoms of 95 cases with SARS-CoV-2 infection. *Gut*. (2020) 69:997–1001. doi: 10.1136/gutjnl-2020-321013
61. Xie C, Jiang L, Huang G, Pu H, Gong B, Lin H, et al. Comparison of different samples for 2019 novel coronavirus detection by nucleic acid amplification tests. *Int J Infect Dis*. (2020) 93:264–67. doi: 10.1016/j.ijid.2020.02.050
62. Wang J, Feng H, Zhang S, Ni Z, Ni L, Chen Y, et al. SARS-CoV-2 RNA detection of hospital isolation wards hygiene monitoring during the Coronavirus Disease 2019 outbreak in a Chinese hospital. *Int J Infect Dis*. (2020) 94:103–106. doi: 10.1016/j.ijid.2020.04.024
63. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet*. (2020) 395:497–506. doi: 10.1016/S0140-6736(20)30183-5
64. Wang W, Xu Y, Gao R, Lu R, Han K, Wu G, et al. Detection of SARS-CoV-2 in different types of clinical specimens. *JAMA*. (2020). doi: 10.1001/jama.2020.3786. [Epub ahead of print].
65. Metlay JP, Waterer GW, Long AC, Anzueto A, Brozek J, Crothers K, et al. Diagnosis and treatment of adults with community-acquired pneumonia. An official clinical practice guideline of the American Thoracic Society and Infectious Diseases Society of America. *Am J Respir Crit Care Med*. (2019) 200:e45–67. doi: 10.1164/rccm.201908-1581ST
66. Yeo C, Kaushal S, Yeo D. Enteric involvement of coronaviruses: is faecal–oral transmission of SARS-CoV-2 possible? *Lancet Gastroenterol Hepatol*. (2020) 5:335–7. doi: 10.1016/S2468-1253(20)30048-0
67. Ding S, Liang TJ. Is SARS-CoV-2 also an enteric pathogen with potential fecal-oral transmission: a COVID-19 virological and clinical review. *Gastroenterology*. (2020) 159:53–61. doi: 10.1053/j.gastro.2020.04.052
68. Zhang Y, Chen C, Zhu S, Shu C, Wang D, Song J, et al. Isolation of 2019-nCoV from a stool specimen of a laboratory-confirmed case of the coronavirus disease 2019 (COVID-19). *China CDC Weekly*. (2020) 2:123–4. doi: 10.46234/ccdcw2020.033

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 28 August 2020
doi: 10.3389/fimmu.2020.02147

What's Sex Got to Do With COVID-19? Gender-Based Differences in the Host Immune Response to Coronaviruses

*Nirupa Gadi[1,2], Samantha C. Wu[1,2], Allison P. Spihlman[1,2] and Vaishali R. Moulton[1]**

[1] Division of Rheumatology and Clinical Immunology, Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, United States, [2] School of Medicine, Boston University, Boston, MA, United States

The novel severe acute respiratory syndrome coronavirus 2, the cause of the coronavirus disease 2019 (COVID-19) pandemic, has ravaged the world, with over 22 million total cases and over 770,000 deaths worldwide as of August 18, 2020. While the elderly are most severely affected, implicating an age bias, a striking factor in the demographics of this deadly disease is the gender bias, with higher numbers of cases, greater disease severity, and higher death rates among men than women across the lifespan. While pre-existing comorbidities and social, behavioral, and lifestyle factors contribute to this bias, biological factors underlying the host immune response may be crucial contributors. Women mount stronger immune responses to infections and vaccinations and outlive men. Sex-based biological factors underlying the immune response are therefore important determinants of susceptibility to infections, disease outcomes, and mortality. Despite this, gender is a profoundly understudied and often overlooked variable in research related to the immune response and infectious diseases, and it is largely ignored in drug and vaccine clinical trials. Understanding these factors will not only help better understand the pathogenesis of COVID-19, but it will also guide the design of effective therapies and vaccine strategies for gender-based personalized medicine. This review focuses on sex-based differences in genes, sex hormones, and the microbiome underlying the host immune response and their relevance to infections with a focus on coronaviruses.

Keywords: coronavirus, SARS-CoV, COVID-19, sex, gender, immune response, infection immunity

OPEN ACCESS

Edited by:
Shen-Ying Zhang, The Rockefeller University, United States

Reviewed by:
Antonio Riva, Foundation for Liver Research, United Kingdom
Sara Louise Cosby, Agri-Food and Biosciences Institute (AFBI), United Kingdom
Juliet Spencer, Texas Woman's University, United States

***Correspondence:**
Vaishali R. Moulton
vmoulton@bidmc.harvard.edu

Specialty section:
This article was submitted to Viral Immunology, a section of the journal Frontiers in Immunology

Received: *15 May 2020*
Accepted: *07 August 2020*
Published: *28 August 2020*

Citation:
Gadi N, Wu SC, Spihlman AP and Moulton VR (2020) What's Sex Got to Do With COVID-19? Gender-Based Differences in the Host Immune Response to Coronaviruses. Front. Immunol. 11:2147. doi: 10.3389/fimmu.2020.02147

INTRODUCTION

Infecting both wild animals and royalty, the novel coronavirus has been able to proliferate and cause the worst pandemic of the 21st century. As the world races to analyze the behavior of this pathogen and develop a therapy for its disease, epidemiological studies have shown a male sex-based bias in disease severity (1, 2) and increased rates of mortality in men over women (**Table 1**). A study of over 70,000 coronavirus disease 2019 (COVID-19) patients in Italy revealed a wide variability in the case fatality rate (CFR). Increasing with age, average rates ranged from 0.16 to 20.88% for women and 0.27–34.68% in men. Overall, men were calculated to have a risk ratio up to 1.74 when compared to women. Of course, sex is intimately tied with demographics and characteristics such

TABLE 1 | Gender-based differences in COVID-19 case and mortality rates.

Country	Cases (%)		Mortality (%)		Total	
	Men	Women	Men	Women	Cases	Deaths
United States	48	52	54	46	5,416,639	170,194
Brazil	55	45	58	42	3,340,197	107,852
India	76	24	73	27	2,647,663	56,757
South Africa	43	57	53	47	587,345	11,839
Peru	56	44	71	29	535,946	26,281
Mexico	53	47	65	35	522,162	56,757
Colombia	53	47	64	36	468,332	15,097
Chile	53	47	60	40	387,502	10,513
Spain	43	57	57	43	359,082	28,646
Iran	57	43	59	41	345,450	19,804
United Kingdom	43	57	57	43	275,200	42,072

Data are collated from the following sources. (1) https://globalhealth5050.org/covid19/sex-disaggregated-data-tracker/; (2) http://www.ijmr.org.in; (3) https://coronavirus.jhu.edu/map.html; (4) https://www.duna.cl; (5) https://www.cdc.gov/mmwr/volumes/69/wr/mm6924e2.htm.

as gender, profession, and hygiene, and there is a plethora of confounding variables between sex and COVID-19 severity. Men are known to smoke more than women and have higher rates of non-communicable diseases, such as type II diabetes and hypertension (1). Meanwhile, women are more likely to work in the healthcare field and therefore have higher rates of nosocomial infection. However, these factors do not deny the fact that physiology may differ dramatically between the sexes, especially in the context of infection.

Across species, females tend to develop a stronger innate and adaptive immune response to contagions. In male and female mice with SARS, male mice had a ∼90% mortality rate, while female mice had a mortality rate of 20%. Doubling the infection load killed every male mouse, while 40% of female mice survived. This overall sex bias was statistically significant and consistent over other strains of mice (3). From an evolutionary standpoint, this increases the reproductive fitness of a species, as mothers are more likely to survive and care for their offspring. Interestingly, parental responsibility is associated with greater immune capability beyond female sex. In seahorses and other fish species, for example, the father is responsible for carrying, delivering, and supporting seahorse fry, and there is an observed upregulation in immunity in these species (4).

Paradoxically, the increased immune function observed among women is accompanied by an increased risk of inflammatory and autoimmune diseases (AD). Women can be 8–9 times more likely to develop an AD compared to men (4). In SARS, these strong immune tactics may inadvertently cause disease through destruction of host tissue. Fortunately, upregulation of proinflammatory immune processes does not seem to occur as much in women with COVID-19. In this case, increased immune function pertains to enhanced anti-inflammatory regulation and antiviral defense (1, 2).

This review will focus on the intrinsic differences in cell types and humoral components of innate and adaptive immunity (**Figures 1**, **2**). Then, the influence of genetics (**Figure 3**), sex hormones (**Table 2**), and microbiome variances across sex will be evaluated. Additionally, the aspects of the ACE2 receptor (**Figure 4**) for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) will be considered, as this unique receptor has multidimensional differences between men and women. Finally, sex differences in the immune response to vaccines will be discussed. Sex is an underappreciated biological variable, and sufficient study and analysis of physiological dimorphisms are necessary to obtain a proper understanding for SARS-CoV-2 interactions in the body.

CLINICAL COURSE

Severe acute respiratory syndrome coronavirus 2 is a novel coronavirus structurally and pathologically related to the original SARS-CoV of 2002. Like its older relative, the new strain of virus accesses host cells using peplomers that are primed by TMPRSS2 to bind the ACE2 receptor (5). The novel coronavirus is an enveloped positive-sense single-stranded RNA virus capable of infecting multiple organ systems in its host, and the density of ACE2 receptors in each tissue correlates with the severity of organ-specific pathology (6). In the lungs, over 80% of cells that express ACE2 are type II pneumocytes, making the lower respiratory tract the most vulnerable target (1). Severe acute respiratory syndrome coronavirus 2 infects these cells and begins processes of viral replication that induce proinflammatory cytokines that recruit components of the innate immune system. The clinical presentations of COVID-19 patients have been heterogeneous, ranging from asymptomatic to respiratory distress to multisystem organ failure and death. One theory to explain this variable response is that the positive inflammatory feedback becomes uncontrolled, resulting in a cytokine storm that can damage host tissue (1, 5). The pathology of COVID-19 disease is characterized by diffuse alveolar damage with fibrin-rich hyaline membranes. Irregular wound healing of the alveoli from the excessive presence of cytokines often leads to thick scarring. This may result in acute respiratory distress syndrome (ARDS) during which patients often require mechanical ventilators, as this lung damage results in restrictive lung disease from fibrosis and pneumonia (1).

ACE2 receptors are also found in extrapulmonary tissues, such as the epithelial cells of the gastrointestinal tract, liver, kidney, pancreas, and olfactory epithelium. They can also be found in cardiomyocytes, pericytes, and fibroblasts of the heart, which may provide a cellular basis for acute myocardial injury. Damage to pericytes and vascular beds may trigger cascades of abnormal clotting, thrombosis, and resultant ischemia that have been noted. Evidence of ACE2 expression in oligodendrocytes of normal brain tissue has also been indicated by RNA-sequencing analyses, which may suggest an explanation for the plethora of neurologic symptoms such as anosmia and ageusia (2, 5). A recent publication reported the clinical characteristics of over a thousand hospitalized adult patients across China. They noted that the most common presenting symptoms were pyrexia (88.7%) and cough (67.8%) (2). Endorsement of congestion, shortness of breath, fatigue, myalgia,

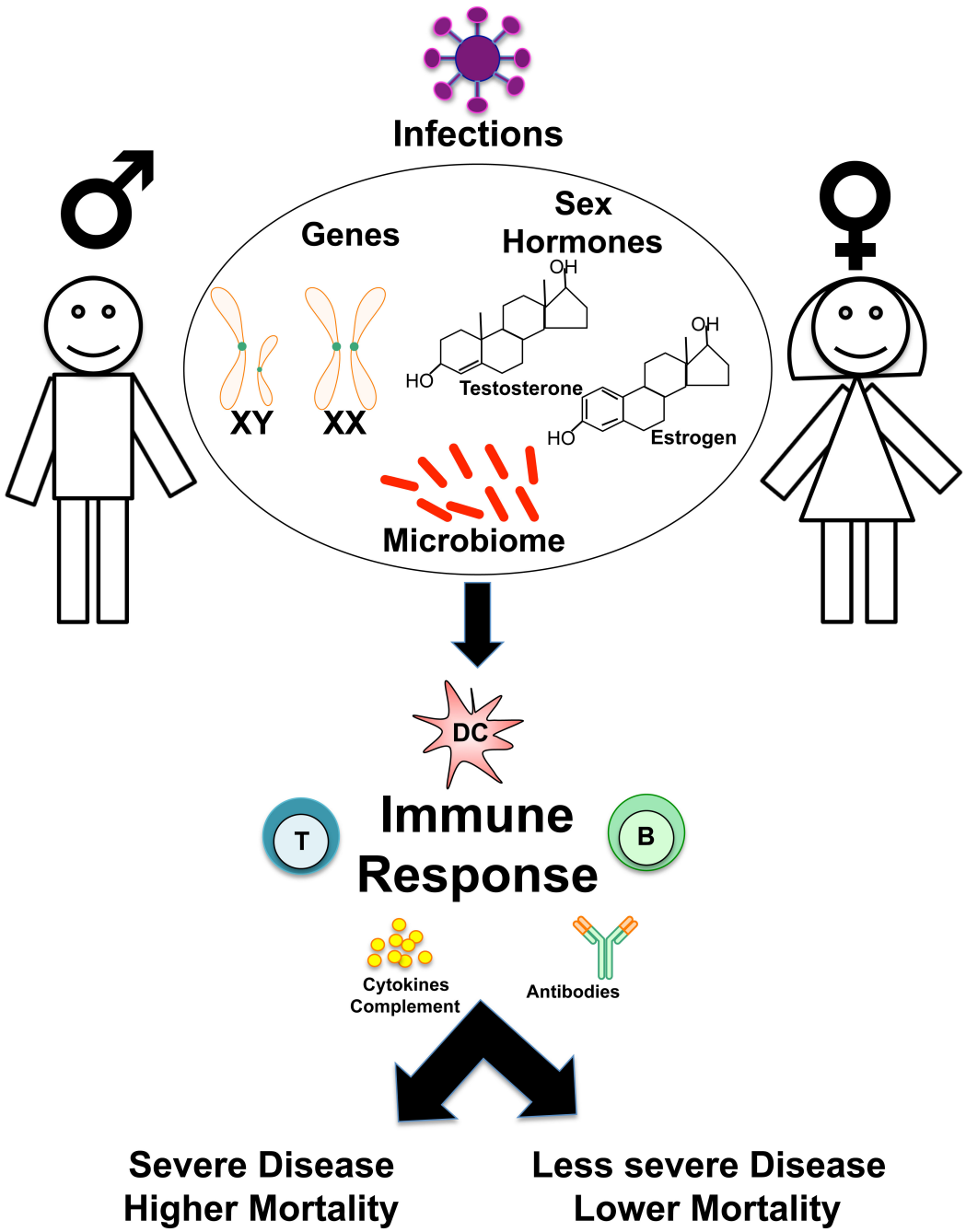


FIGURE 1 | Overview of sex-based differences in the immune response to Infections. Schematic shows how genes, sex hormones, and microbiome may influence sex-based differences in the host immune responses to infections, determining susceptibility, disease course, and clinical outcomes.

headache, and confusion have also been commonly reported in other studies. Emesis, diarrhea, and other gastrointestinal symptoms are also observed in some patients. Brigham & Women's COVID-19 resource hub reported anosmia and/or ageusia in up to 70% of patients[1]. The complete blood count (CBC) on admission was most notable for lymphocytopenia in 80% and thrombocytopenia and leukopenia in about 1/3 of patients (2). There is a mild hepatocellular injury pattern with AST/ALT ratio ~200. Elevated d-dimer, CRP, LDH, CK, ferritin, and other markers of inflammation are also commonly reported. Radiographic findings most commonly included atypical pneumonia with bilateral, peripheral, and posterior features. Chest CT findings include ground-glass opacities and bilateral consolidation in more than half of

[1]https://covidprotocols.org

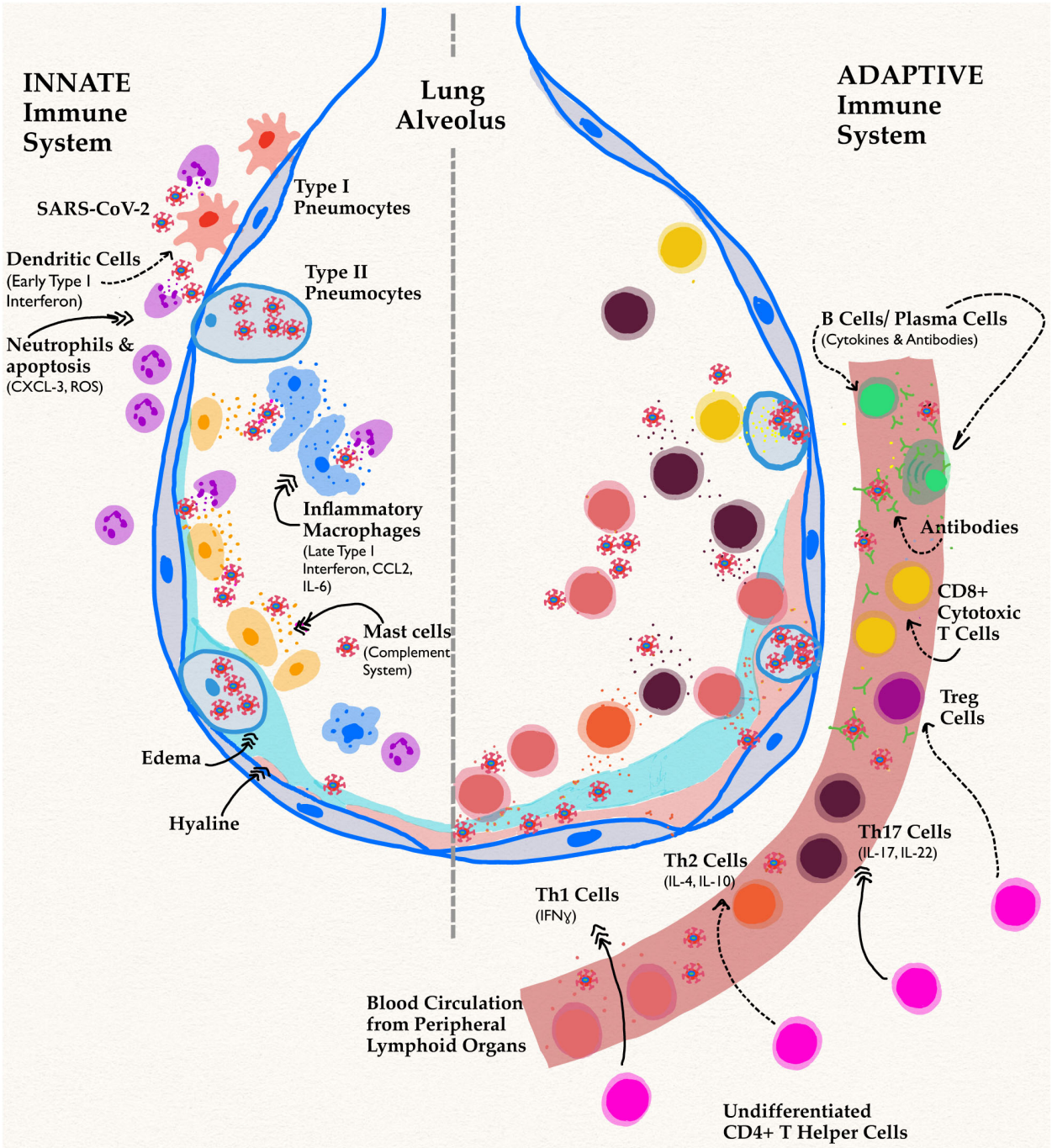
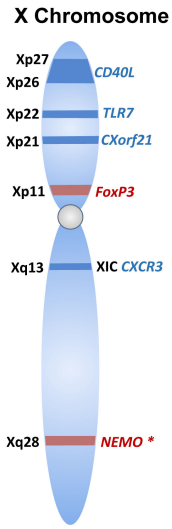


FIGURE 2 | Innate and adaptive immune responses in SARS-CoV-2 Infection. Schematic shows a lung alveolus and a blood vessel. The left side of the figure describes effects of the innate immune system specifically in men, while the right side depicts their delayed adaptive immune functions. Dotted arrows represent a decrease in function or activity, while triple headed solid arrows signify an increase compared to infection in women. The most implicated cell types and their associated cytokines have been shown. In men, the novel coronavirus infection has reduced activation of APC and early proinflammatory processes. A decrease in this pathway causes delayed, excessive proinflammatory cytokine responses leading to the infamous cytokine storm implicated in both components of immunity.

admitted patients (2). Patients with features of these abnormal laboratory findings were most likely in cases of severe disease. Although a plethora of information has been published recently on symptoms, signs, and pathology in COVID-19 disease, little information has been stratified by sex differences. Optimistically, cognizance that these dimorphisms exist will give rise to novel research and therapies that take advantage of these differences.



Chromosome	Gene	XCI Escape	Primary Immune Effect	Mechanism
X	CD40L	Yes	Adaptive	CD4+ T cell proliferation
	TLR7	Yes	Both	PAMP recognition
	CXorf21	Yes	Both	Lysosomal pH optimization
	FoxP3	No	Adaptive	Treg regulation
	CXCR3	Yes	Adaptive	T and B cell trafficking
	NEMO *	No	Both	NF-kB activation
6	C4	No	Innate	Complement system - classical pathway
	HLA	No	Adaptive	T cell antigen presentation

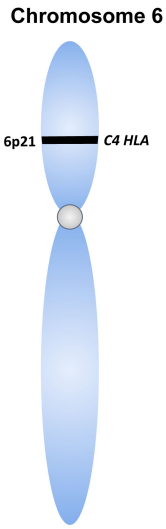


FIGURE 3 | Immune-related Genes implicated in the sex-based differences in the immune response. Schematic shows genes encoded on the X-chromosome and chromosome 6. Table lists the role of these genes in innate and/or adaptive immunity and mechanism of action. Genes indicated in blue text escape X chromosome inactivation, while those that do not undergo escape are indicated in red. NEMO is marked with an asterisk (*) to specify skewed X chromosome inactivation.

IMMUNE RESPONSE

Innate Immunity

Antigen-Presenting Cells

Dendritic cells (DC) are the primary antigen-presenting cells (APC) of the human body and are divided into myeloid or lymphoid types based on their visual characteristics. Lymphoid DC are better known as plasmacytoid dendritic cells (pDC) and are the most potent producers of the type I interferon (IFN αβ). Plasmacytoid dendritic cells are activated by several pattern recognition receptors (PRR), of which the most relevant to the topic is Toll-Like-Receptor 7 (TLR7) (7). This PRR is primarily expressed in pDC and recognizes ssRNA sequences, making it vital for detection of coronaviruses in the host (7–9).

The role of IFNα as it pertains to viral infections and SARS in particular has been hotly contested. Many studies show that IFNα has a protective role in Coronaviridae and other viral infections, and its study as a therapy is promising; on the other hand, there is equal literature to show that it is elevated and responsible for adverse host outcomes, such as fibrotic findings in SARS patients (10–12). The current theory behind these conflicting findings is that the timing and level of IFN release is critical to its effect on the host. In a mouse model, Type I IFN (IFNαβ) administration one day after infection seemed to protect mice from adverse outcomes, while delayed IFN exposure enhanced lethal proinflammatory processes (12). Plasmacytoid dendritic cells are responsible for coordinating an early IFN signal, which seems to be associated with better outcomes.

The first major wave of IFNα for antiviral processes is coordinated by TLR7 signaling to pDC (13). This mechanism has been studied to reveal sex-based differences in signaling. For example, pDC from women are found to produce more IFNα when stimulated by TLR7 than compared to men (7). To compound this effect, TLR7 is a receptor that is encoded on the X chromosome and is able to escape X inactivation, meaning that XX females and XXY men [Klinefelter Syndrome (KS)] have higher expression of TLR7 (9). Coronavirus infection magnifies this sex bias, as recent literature has yielded information that SARS coronavirus is a poor inducer of IFN, as its papain-like protease is capable of inhibiting TLR7 signaling to pDC (8). In females, the increased production of IFNα with the enhanced presence of TLR7 is correlated with greater induction of pDC/TLR7-mediated pathways and immune response, suggesting that coronavirus inhibition of host antiviral pathways is reduced (8, 9).

Granulocytes

Neutrophils

In one study, male and female mice were both infected with SARS-CoV, and their sex-specific outcomes were studied. Male mice had higher rates of vascular leakage, leading to alveolar edema, and a study of bronchoalveolar lavage fluid 3 days after infection showed they had 4–5x higher rates of neutrophils compared to female mice. Neutrophils are vital for a protective immune response, and completely depleting neutrophil populations by use of an αPMN antibody resulted in a 28% mortality compared to a control (3).

However, maintaining a balance in the level of PMNs is critical, as PMNs can also cause pathological states in the host. In the same study, an overly increased presence of PMNs in a coronavirus rat model was found to be correlated with lung tissue inflammation, epithelial cell permeability, and hemorrhagic lesions (14). Additionally, compared with females, male rats' PMNs, showed significantly higher recruitment of CXCL-1, which recruits neutrophils for killing microbes as well as activating protease and reactive oxygen species (ROS) (11, 15).

TABLE 2 | Sex Hormones and their effects on immunity and relevance to COVID-19.

Hormone	Immune Cell/Cytokine	Effect	Relevance to COVID-19
Estrogen	Type 1 IFN	Promotes synthesis	Proinflammatory, beneficial early on but harmful when delayed
	IL-12	Promotes synthesis	Th1 cytokine, proinflammatory
	IL-6	Promotes synthesis	Pro-inflammatory (cytokine storm)
	IL-1β	Promotes synthesis	Pro-inflammatory (cytokine storm)
	Neutrophils	Delays apoptosis	High recruitment and subsequent apoptosis are found in severe patients
	B cells	Promotes activation, maturation, differentiation, Ig antibody production	Beneficial IgG response but cytokine response is higher in women
	CD4 +	Promotes activation, Th1 differentiation	Different T cell types are needed for successful infection control
	Th17	Suppresses response	Th17 is proinflammatory, decreased levels means less host damage
	CD8 +	Increases activity	High levels early on may confer benefit
	Tregs	Increases FoxP3 expression and Treg production	Tregs suppress Th1 and Th17 responses and are anti-inflammatory
	IL-10	Promotes synthesis	Anti-inflammatory, suppresses cytokine synthesis and MHC expression
Progesterone	IL-1β	Suppresses activation	Th1 cytokine, pro-inflammatory
	TNF	Suppresses activation	Pro-inflammatory, neutrophil and endothelial cell immune activation
	T cells	Decreases proliferation	May control T cell responses and cytokines
	IL-4	Increases production	Th2 cytokine, promotes Ig response controls T cell proliferation
	Tregs	Increases production	Tregs suppress Th1 and Th17 responses and are anti inflammatory
	Th17	Decreases production	Protects the host from adverse immune response
	CD8 +	Reduces IFN-γ production and cytotoxicity	Allows higher numbers of these cells without excess proinflammatory cytokines
Testosterone	TNF	Decreases production	Pro-inflammatory, neutrophil and endothelial cell immune activation
	IFN-γ	Decreases production	Pro-inflammatory, activates macrophages and increases antibody response
	IL-10	Increases production	Anti-inflammatory, suppresses cytokine synthesis and MHC expression

Table summarizes the role of sex hormones on immune cells and cytokines and the potential relevance to the SARS-CoV-2 infection.

It seems that in rats, male PMNs have higher rates of apoptosis compared to females, and apoptosis recruits phagocytic cells to the site of cell death (16, 17). Increased damage in the lung tissue of male mice with the presence of up to 500% more PMNs therefore suggests that the neutrophil immune response is pathological in males compared to females, who exhibited few alveolar edema on histological examination.

Eosinophils

The eosinophil response in SARS is largely unexplored, especially as it pertains to sex differences. There is, however, convincing evidence that female sex hormones are strong activators of eosinophils. Estrogen promotes eosinophil development, adhesion, and degranulation. Eosinophil numbers spike when female rats have higher estrogen levels, and surgical excision of rat ovaries results in a sharp decrease in uterine eosinophils (17). A genetic study of patients who experienced severe SARS-CoV symptoms revealed that the gene for the enzyme eosinophil-derived neurotoxin (EDN) is expressed more in healthy individuals than in people severely afflicted by SARS (18). Eosinophil-derived neurotoxin is a key protein found in eosinophil granules and has strong ribonuclease activity especially when activated by proinflammatory stimuli. Differences in EDN expression have not yet been analyzed in males vs. females of any species, but if female sex hormones are supportive of eosinophil development and granulation, women expressing EDN may have a unique advantage in fighting SARS infection (12).

Basophils

Basophils are the rarest of the granulocytes, and study of the basophil response to coronaviruses is even more unknown than eosinophil interactions. In one study, the incubation of human basophils with mild strains of coronavirus did not cause the leukocytes to degranulate or release histamine (19). More studies are needed, however, to establish that basophils are not involved in the coronavirus response or that there are no sex-related differences in their interactions.

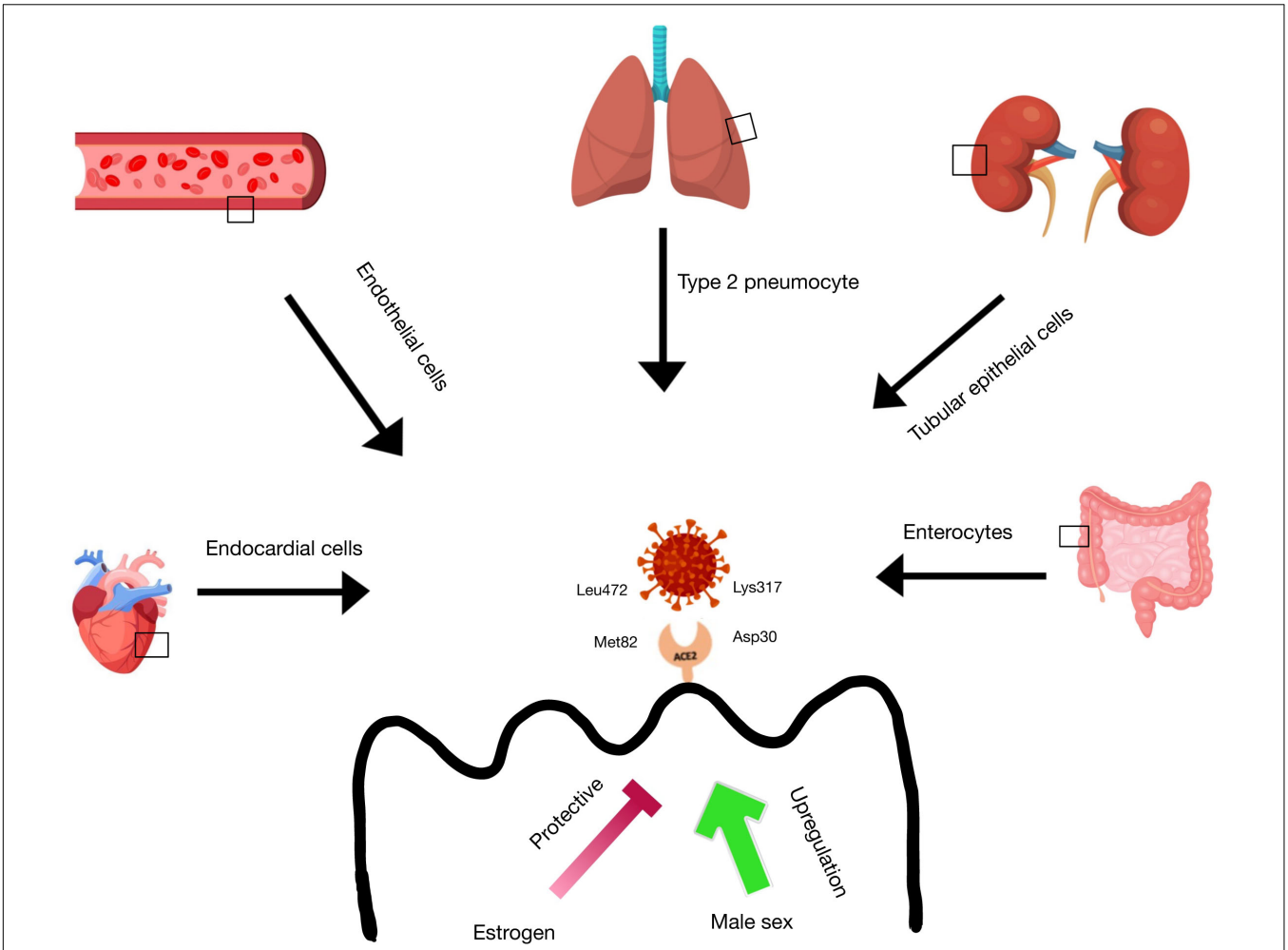


FIGURE 4 | ACE2 receptor expression in tissues. ACE-2 receptor expression is found in the lung type 2 pneumocytes, heart endocardial cells, blood vessel endothelial cells, kidney tubular epithelial cells, and intestinal enterocytes. There is evidence that estrogen has a protective function on ACE-2 receptor expression in these tissues, while male sex is correlated with an upregulation of ACE-2 receptors in these tissues. SARS-CoV-2 is able to bind to the ACE-2 receptor via ionic interactions between Lys317 on the virus and Asp30 on the receptor and via Van der Waals interactions between Leu472 on the virus and Met82 on the receptor.

Mast cells

Mast cells reside in the submucosal layers of the respiratory tract; although they are mainly known for their functions in allergy responses, mastocytes are also intimately involved in protection from viral invaders (20). In fact, their roles and activation in the immune response are incredibly interesting in the context of sex bias. It seems that, although mast cells from female mice bear granules with higher enymatic activity, these cells are activated to a lesser degree via the complement system than mast cells from male mice. This may explain the increased preponderance of lung injury in men with coronavirus infection. Even when controlling for differences in number and extent of activation, mast cells from female mice make, store, and secrete more histamine than mastocytes of male origin. With binding to high-affinity IgE receptors (FcεR1), mast cells from female mice release an increased amount of other preformed proinflammatory mediators, such as tryptase, chymase, and TNF-α (17, 21). Gene analysis study has shown that genes such as *Tnf, Hexa,* and several *mcpt* genes that encode these intracellular granule mediators are upregulated in female mice. Mast cells from women store this increase in protein product by increasing the packing density of granules in mast cells (22). This set of studies was repeated in the presence of various levels of female sex hormones, as previous literature has shown that the menstrual cycle can affect properties of mast cells in rodent models. The study found that there was no statistically significant difference in the amount of histamine that mast cells released across levels of sex hormones in male and female mice (21). This shows that while previous studies may point to significant hormonal effects on mast cell properties, there are sex-based differences in mastocyte biology that are not attributable to hormone interaction.

Mastocytes in the context of SARS-CoV infection are intimately involved with the complement system, which has significant proinflammatory responses that can result in pathological states. In fact, coronavirus infection activates

the classical, the lectin, and the alternative pathways of the complement system. Of particular interest are C3 and C5 proteins which activate mast cell degranulation in SARS to trigger a cytokine storm (19, 23). This cytokine storm can lead to further downstream effects such as hyperemia and vascular permeability, which can result in acute, fatal lung injury. C5a levels are actually predictive for ARDS development, and blockade of the C5a pathway in MERS infection reduces lung injury in mice (20). In a similar vein, inhibition of the complement cascade via inhibition of C3 in M15-infected mice results in less recruitment of neutrophils and inflammatory monocytes to the lung tissue (23).

The conclusion that reduced activation of the complement cascade results in maintenance of healthy lung tissue correlates to the sex bias found in levels of complement proteins. A recent study of 50,000 racially diverse subjects found that, compared to men, women have significantly lower levels of C4, an activator of C3. They were also able to prove that this results in a 42% decrease in C3 in women compared to men (22). In an investigation of Caucasian populations, levels of C3, C5, C7, C8, and C9 were significantly lower in women compared to men. Women had about 53% lower C5 protein levels than men. Also women had lower amounts of proinflammatory positive regulators of the cascade such as properdin, mannan-binding lectin (MBL), and Ficolin-3 (24). These reduced levels of complement proteins were found to control activity of the whole cascade and its terminal products for the classical, lectin, and alternative pathways that are involved in SARS infection (20, 24). While these findings were obtained from a Caucasian cohort, evaluation of these in other race/ethnicities remain to be seen. Yet, from both studies it is justifiable to say that the female host has mechanisms to reduce proinflammatory effects even with more potent mastocytes. In the context of SARS, these techniques may prove useful if they are an explanation for the minimized incidence of pulmonary injury.

Monocytes/Macrophages

In the discussion of pDC, it was iterated that the role and clinical effects of Type I IFN are still incompletely understood and are often conflicting across various studies. Current theories support the idea that initial secretion of Type I IFN is effective at reducing viral load without causing damage to the human host in which XX females and XXY males seem to have better outcomes than XY males in a mouse SARS-CoV model. For example, Type I IFN administration 6 h post-infection (before viral peak) in mice infected with SARS coronavirus completely protected them from clinical disease (10, 12). Elevated and extended exposure of the host to IFN, however, led to excessive proinflammatory pathways and pulmonary pathology. In the same study, mice were exposed to IFN post-peak of viral titers which resulted in lethal pathology. This acute lung injury in mice and humans with SARS is characterized by the presence of IFN-stimulated inflammatory monocyte-macrophages (IMM) and their associated proinflammatory cytokines in bronchoalveolar lavage fluid (10–12). Although women have higher activation of IFN pathways early in coronavirus infections, it is unknown if there is a difference in IFN levels between men and women after peak viral load. It may be that early activation of antiviral pathways more effectively reduces viral load by priming the innate and adaptive immune systems. This early activation can better protect the host from the cytokine storm found mostly in males that is associated with later IFN secretion (10, 12, 25).

Although the sex differences regarding late Type I IFN levels are unknown, SARS-CoV-infected male mice have higher rates of IFN-stimulated IMM in their bronchoalveolar lavage fluid. Just three days post infection, there were 2–3-fold greater numbers of these IMM (11). Cytokine analysis showed that IMM release from males had a higher frequency of the pro-inflammatory cytokines CCL2 and IL-6. IL-6 is an activator of CCL2 binding to CCR2 which promotes lymphoid and myeloid chemotaxis as well as properties of leukocyte adhesion, polarization, secretion, and survival in the immune responses (12, 26). However, because this IFN-stimulation of IMM is uncontrolled in SARS patients with poor outcomes, methods of reducing their potency are advantageous. Ablation of IFNAR, a Type I IFN receptor, 3 days into infection produced improved outcomes from coronavirus infections (10). Additionally, use of monoclonal antibodies has also been considered for therapy in humans. In SARS-CoV infection in mice, MC21 antibodies could bind to CCR2, and this competitive inhibition provided significant protection in male mice prone to poor-outcomes. This study demonstrated that a depletion in IMM signaling is a protective feature in SARS-infected female mice (11).

Natural Killer Cells

Natural Killer (NK) cells are cytotoxic lymphocytes of the innate immune system that target cancerous and infected cells. Their exhaustion is correlated with disease progression (27), as they are primary secretors of IFN-γ, TNF-α, colony stimulating factors (CSF), and many other cytokines (15, 27). In the diseases caused by both SARS-CoV and SARS-CoV-2, studies have shown that there is a marked decrease in the total number and activity of NK cells in infected patients. In the same vein, there are significantly lower recorded amounts of CD107a+ NK, IFN-γ+ NK, IL-2+ NK, and TNF-α+ NK in COVID-19 patients, suggesting that functional exhaustion of NKC is associated with coronavirus infection (27).

There is a plethora of puzzling information about the sex bias in NK cells. Some studies have found greater numbers and activity of NK cells from male rodents compared with females (4, 17). Another study of healthy geriatric individuals found that while there was an initial surplus of NK cells in men less than 70 years of age, there was a steep increase and superiority in NK cell function in women over 70 years (17, 28). This post-menopausal finding seems to defy other studies that found a rise in NK cells during the periovulatory phase when estrogen and progesterone are increased (4, 29). It is possible, however, that even if estrogen derivatives increase the number of NK cells, the cytotoxic capabilities of these cells may be reduced.

Adaptive Immunity

CD4 T Cells

Th1/Th2 cells

The physiology of CD4 T helper type 1 (Th1) and 2 (Th2) cells in COVID-19 infection is the most striking when considering the implications for sex differences. Th1 cells are

differentiated CD4+ T lymphocytes that are microbicidal and proinflammatory. In contrast, Th2 cells produce cytokines that are more anti-inflammatory and redirect focus to humoral immunity mechanisms. The homeostasis between Th1 and Th2 cells is vital for the obliteration of infectious microbes without causing pathological states in the host. The Th1/Th2 ratio is paramount for host success, as extermination of distinct pathogens will require different levels of activation in Th1 and Th2 lymphocytes (30–33). An improper ratio of Th1/Th2 cytokines may result in prolonged disease states such as in lepromatous leprosy or tuberculosis.

The SARS coronavirus of 2002 is notable in that it exclusively produced an activation of Th1 cells and cytokines in the host. In a study of patients in China, the Th1 response produced a significant elevation in the proinflammatory cytokines IFN-γ, IL-1β, IL-6, and IL-12 (30). These protein levels were elevated for at least 2 weeks after onset and were sufficient for full recovery of the host. Across several studies, induction of anti-inflammatory Th2 pathways was not necessary for host survival (30–32). The novel coronavirus SARS-CoV-2, however, seems to work differently when compared to its older relative, as sufficient induction of anti-inflammatory Th2 response is necessary. Patients with COVID-19 show activation of both Th1 and Th2 pathways over the course of infection with significant levels of IFN-γ, IL-1β, and IL-6 as well as IL-4 and IL-10, respectively (31, 32). An elevated Th1/Th2 ratio has been correlated to increased risk of mortality in COVID-19 patients as the proinflammatory cytokine IL-6 has been predictive for severe lung pathology (31). Another COVID-19 study found that aberrant Th1 cells expressing significant levels of IFN-γ, IL-6, and GM-CSF were found only in intensive care unit (ICU) patients. Levels of these cytokines were much lower in non-ICU patients and the control group (32), indicating that pathogenic Th1 cells correlate with the hyper-inflammatory response in SARS-CoV-2 pathogenesis.

To the best of the authors' knowledge, there is no literature yet on sex differences in Th1 and Th2 responses to COVID-19. However, the implications of the Th1/Th2 balance could be important in pregnant women with SARS-CoV-2 infection. Physiologic changes within the immune system during pregnancy typically involve an attenuation of the Th1 response and a shift toward Th2 anti-inflammatory pathways (31, 32). This is in the interest of protecting the developing fetus from an overactive cell-mediated immune response, while simultaneously developing antibodies for passive transfer of immunity through the placenta and breast milk. In the Th1-dominant SARS-CoV infection, the CFR of pregnant women was estimated to be up to 18%, as the maternal immune system would attenuate Th1 and inadvertently compromise itself. The original SARS-CoV stands in contrast to the novel SARS-CoV-2 COVID-19, however, where the CFR is ∼1% (34); although hospitalization, ICU, and ventilation requirements were higher in pregnant compared to non-pregnant women, the risk of death was the same compared to non-pregnant women (35), and disease course appears to be milder than in SARS-CoV and MERS (31). Although there may be numerous variables at play, it seems that host survival in COVID-19 is tied to substitution of the proinflammatory Th1 for the anti-inflammatory Th2 that is dominant in gravid hosts.

Th17 cells

Th17 cells are a differentiated form of CD4+ T lymphocytes that mainly produce IL-17, IL-22, IFN-γ, and GM-CSF. They are also involved in the production of related proinflammatory cytokines such as IL-6, IL-26, and TNFα (36–38). It was found that the severity in MERS and both forms of SARS was positively correlated with Th17 and IL-17 levels in patients. IL-17 is the most well-studied cytokine of Th17 cells, as it is the lymphocyte's hallmark cytokine. In coronavirus infections, IL-17 encourages the assembly of downstream proinflammatory cytokines that result in activation of neutrophil chemokines and secretory elements that damage lung parenchyma (37). An investigation of polymorphisms for IL-17 genes in SARS patients showed that individuals predisposed to lower levels of IL-17 activation had significantly increased 30-day survival when compared with patients prone to increased IL-17 production (36). Th17 lymphocytes also contribute to ARDS pathogenesis by activation of IL-22, which seems involved in the production of mucin and fibrin-rich secretions in patients with pulmonary edema (36, 38).

While the role of gender in relation to Th17 function in coronavirus infections has yet to be studied, there is some literature on how this cell type affects AD. In AD that predominantly affect males, there has been significant literature on Th17 cells playing a paramount role in disease progression (37–39). For example, although multiple sclerosis (MS) affects 2–3 times more women than men, men tend to experience more rapid and aggressive disease progression (39). Similarly, in systemic lupus erythematosus (SLE) which afflicts women 9–10 times more often than men, men tend to experience more severe complications especially nephritis leading to renal failure. In a mouse model of MS, Th17 cells from male mice were transferred into female mice and found to induce significantly higher levels of IL-17 and IFN-γ secretion that led to worsening AD and pathology (38). This finding suggested that male sex is a crucial and inherent element of disease-induced severity related to Th17 cells. Whether this finding is applicable to host attack in the context of SARS remains to be determined.

Treg cells

Regulatory T (Treg) cells are a subset of CD4 T lymphocytes with unique immunosuppressive activities. As discussed previously, the homeostasis between proinflammatory/anti-inflammatory processes is critical to clearance of an infection without damage to the host and subsequent AD (40). The optimal balance of these activities is different for every pathogen, but, in the context of mouse hepatitis virus (MHV) coronavirus, Tregs are necessary for mild disease outcomes, as their depletion results in increased mortality (40, 41). While studies of Treg influences and functions have been performed on several types of respiratory viruses, there is little information on their roles against human coronaviruses. As seen in the disparity between Th1/Th2 responses with both SARS coronaviruses, adaptive immunity mechanisms may vary significantly even between similar virus strains. The role and activity of Treg lymphocytes, therefore, cannot yet be verified in the context of COVID-19, but these cells may offer a benefit by reducing excessive damage to lung parenchyma.

The transcription factor Foxp3 serves not only as a marker for Treg cells, but it is also necessary for their development, maintenance, and suppressive functions (42, 43). Foxp3 is encoded on the X chromosome and can escape X inactivation, giving XX females higher activity of Foxp3 and Treg cells (33, 41). This may give women an immunosuppressive advantage in the context of coronaviruses. Scarcity of Treg cells from loss-of-function alterations in *Foxp3* lead to severe and even lethal inflammation in human and rodent models of coronavirus infection (41). There is more literature on this appreciable sex bias of Treg cells that will be expanded on in the "Genetics" section of this article.

CD8 T Cells

CD8+ cytotoxic T cells are differentiated lymphocytes that kill infected, cancerous, or damaged cells through recognition of antigens presented via major histocompatibility complex (MHC) class I and consequent signaling. Across several studies, there are a significant portion of COVID-19 patients who present with lymphopenia and markers of T cell exhaustion. PD1 and Tim3 are molecular markers of this fatigue in CD8+ cells and are elevated with lower levels of circulating cytotoxic T cells. It was found that as patients progressed from prodromal to active symptoms, their levels of PD1 and Tim3 would directly increase. In the same vein, patients in the ICU had much higher expression of these markers compared to non-ICU and control populations (44, 45). Another study used granzyme B and perforin markers of induced apoptosis as indicators of CD8+ cell fatigue. Consistent with previous work, these markers were significantly elevated in critically ill patients compared with those who were only mildly symptomatic with COVID-19 (46). This new data indicates that SARS-CoV-2 promotes extreme stimulation and ensuing exhaustive collapse of CD8 + T cells, which is similar in fashion to many malignancies (44–46).

Causes and effects of lymphopenia have not been thoroughly studied in SARS, and much less can be said for differences in sex. Healthy females in general, however, exhibit higher cytotoxic T-cell activity along with an upregulation of CD8+ genes (47), many of which have estrogen response elements (ERE) in their promoters. Also, as discussed earlier, women have greater activation of TLR7 and pDC. It appears that upregulation of this pathway results in higher levels of CD8 + T-cell activation in women compared to men (7, 13, 47). The implications of greater CD8+ function are unknown in the context of this novel virus, but it may decrease viral load in early coronavirus infection (47).

Memory T Cells

After an initial encounter with a pathogen, antigen-specific naive CD4 and CD8 T cells clonally expand to become effector T cells, which mount a cellular and humoral response against the offending pathogen. After pathogen clearance, most effectors die by apoptosis, while some survive and persist to become long-lived memory T cells, and it is these cells that offer the host protection from subsequent encounters with the pathogen. Memory cells also form the basis for vaccinations which elicit a similar immune response albeit at a significantly reduced magnitude without causing disease. Memory T cells are heterogeneous in phenotype, function and localization and include central memory (Tcm), effector memory (Tem), tissue resident memory (Trm), terminally differentiated memory (Temra), and other cells (48). These antigen-specific cells are the basis of vaccine development, as they are skilled in triggering a targeted immune response upon re-exposure to an antigen. As SARS-CoV-2 is a novel infection, development and viability of memory T cells in men and women is truly unknown, especially in the face of viral mutation.

As SARS-CoV-2 is a novel infection, development and maintenance of memory T cells in men and women is truly unknown, especially in the face of viral mutation. Several studies on the original 2002 SARS-CoV have, however, shown that SARS-CoV-specific CD8+ memory cells can be found 6–11 years later in the blood of past patients, while specific CD4+ cells have only been reported for up to 2 years (48–51). In support of a gender difference in memory T-cell survival, it appears that healthy women harbor greater numbers of CD4+ lymphocytes (52). In SARS, however, CD4+ T cells have mainly exhibited a central memory phenotype while CD8+ cells were of the effector memory phenotype, suggesting that CD8+ memory cells may be dominant in host attack upon re-exposure (51). This also suggests that CD4+ abundance in women may not affect long-term and effective resistance to SARS. In anamnestic response of CD8+ cells to the SARS coronavirus, these lymphocytes proved valuable in that they could produce cytokines such as IFN-γ, TNF-α, IL-2, and granzyme B to reduce lung viral loads in mice (49–51). A study did, however, find that while CD8 + cells were functional in protecting vulnerable hosts from reinfection, their pathways were downregulated without SARS CoV-specific CD4+ T cells or differentiated B cells (49).

While memory T-cell immunity may be the key to protect from reinfection from SARS-CoV, SARS-CoV-2 has shown evidence of mutation and therefore carries the possibility of reinfection. Vaccine development has been rapidly under way since the novel coronavirus's introduction, and recommendations based on genome analysis have been made to target portions of the virus that are least likely to mutate. A promising population coverage analysis found that 23% of known SARS-CoV-2 epitopes map parallel to SARS-CoV, and no alterations have been reported in these epitopes among known SARS-CoV-2 genomes (53). This strongly suggests their potential for inducing a viable T-cell response against SARS-CoV-2.

B Cells

Antibody functions

Antibody secretion is the primary function of B lymphocytes. In a study of almost 300 patients who had SARS-CoV-2, 94% formed IgM antibody titers, and 100% developed IgG at a median of 13 days post symptom onset (54). As expected, IgG antibody levels were stable while IgM antibodies reached low levels within 5 weeks and became undetectable at 7 weeks (55). Importantly, the IgM response typically precedes IgG, but several investigations have seen synchronous seroconversion of IgG and IgM as well as appearance of IgM or IgG first at about equal rates (54, 55). This may have to do with the upregulation of IL-4, IL-10, and other cytokines promoting antibody class-switching at an ultra-rapid rate (56). These interleukins are part of the Th2

pathway which is naturally enhanced in women, suggesting that there may be a physiological sex difference in antibody switching, although no known studies have been done in this specific area (32) (34). Another study investigated IgG levels between male and female COVID-19 patients, stratifying patients by disease severity into mild, moderate, and severe status and into early, active, and recovery phases. While there was no significant difference in serum IgG levels across gender in mild and recovering patients, IgG levels in women were significantly elevated in the early disease phase and in severe cases (57). This situational increase in antibody titers cannot yet be determined as helpful or harmful in SARS, but it is worth noting for future investigation.

Cytokine functions

B-cell production of cytokines make these lymphocytes powerful regulators of adaptive immunity, but in many cases turn maladaptive, such as in SARS. A series of seven case studies was recently published on B-cell immunocompromised COVID-19 patients and sheds light on the excessive lymphocyte immune response. Disease presentation of patients with common variable immune deficiency (CVID) hypogammaglobulinemia were compared to patients with agammaglobulinemia (AGG). Surprisingly, the AGG cases proved to be mild, and the patients had normal lung CT scans with no consolidation. The patients were treated in the hospital for a maximum of 3 days and went home without requiring assisted ventilation. In contrast, the CVID patients had extensive ground glass opacities and alveolar consolidation on lung CT. They were in the hospital for at least two weeks and needed antiretroviral therapy (ART), IL antagonists, and some required mechanical ventilation. These findings suggest that complete depletion of B cells is associated with mild symptoms in SARS. According to the study, the difference in patient outcomes was likely due to the lack of non-Ig B cell cytokine functions, meaning that patients with AGG avoided a host-harming cytokine storm. Based on these results, inhibiting B-cell cytokines may be a useful tactic in both men and women with COVID-19 (47).

This cytokine activation is linked to stimulation of TLR in T-cell independent activation (46). Blood analysis of COVID-19 patients has shown marked decreases in naïve B cells with increases in plasma cells, suggesting that there is a pressure on naïve B cells to mature and proliferate for increased humoral immunity. In the context of coronaviruses, IFN-α is released by pDC upon stimulation from TLR7 binding. This causes an upregulation of TLR7 receptors on the surface of the naïve B lymphocyte. This process authorizes B cells to respond to activation from coronavirus-TLR7 binding by immediate expansion and differentiation without much T cell interaction. These mature and activated B cells are capable of producing IgM as well as proinflammatory cytokines, namely IL-6. IFN-α is also able to induce this effect in memory B cells which are activated to produce IgM and IgG. Together, these data indicate that pDC IFNα controls the proliferation and differentiation of B lymphocytes into Ig-secreting plasma cells. This conclusion is paramount in coronavirus infections, as women express more TLR7, initially secrete more IFN-α, and therefore have greater activation of B-cell antibodies and cytokines (45, 46). Contrary to other immune cells (discussed above), it seems that B-cell-derived cytokines may be more harmful to a female host.

Genetics

Women mount a stronger immune response against viral infections than men (58). Women possess both maternal and paternal X chromosomes, which necessitates the silencing of one copy of genes in order to ensure an appropriate gene dosage. The silencing of one copy, or X chromosome inactivation (XCI), leads to functional mosaicism in women with regards to X-linked genes (58). The X inactivation center (XIC) is located at locus Xq13 (58). X chromosome inactivation is cell-specific and variable among individuals, which causes some cells to express the maternal chromosomal copy and others to express the paternal copy. In turn, this leads to a diversity of possible immune responses in females, which provides women with a wider variety of tools with which to fight pathogens (59). Skewed inactivation patterns may additionally offer a protective effect by silencing immunodeficiency-causing mutations (60). Furthermore, X-chromosome skewing may preferentially express beneficial alleles, leading to a larger proportion of cells producing functionally advantageous gene products (60). X chromosome inactivation is particularly relevant to discussion of the SARS-CoV-2 immune response, as the X chromosome encodes for several genes involved in both adaptive and innate immunity, including those involved in the TLR pathway (58). Cellular mosaicism suggests that women may be better equipped to respond to immune challenges, particularly viral infections such as SARS-CoV-2.

Several genes fail to undergo XCI and thus "escape" inactivation, leading to biallelic gene expression with a double dosage and resulting differences in gene dosage between sexes. About 15% of X-linked genes escape XCI, while 10% are only partially inactivated (59). Evidence shows that XCI escape commonly occurs in female lymphocytes, which display atypical heterochromatic modification and tend to reactivate the inactivated X chromosome (Xi) (61). Expression of X inactive specific transcript (XIST) RNA has been observed in both B and T cells, leading to biallelic expression of CXCR3, TLR7, and CD40L (58, 61). The XCI escape of genes involved in the immune system may further contribute to an immunologic advantage in women.

CXCR3

Chemokine receptor CXCR3, located at locus Xq13, is one such gene that escapes inactivation (58). *In vitro* mouse studies of CXCR3 expression confirm that females express both copies of CXCR3. Biallelic CXCR3+ T cells yield more CXCR3 protein and subsequently secrete more IFN-γ, IL-2, and CD69 than monoallelic CXCR3+ T cells (62). CXCR3 functions to mobilize NK cells and T and B lymphocytes to areas of inflammation and may aid in effector Th1 cell differentiation (63, 64). The role of CXCR3 in leukocyte recruitment and the Th1 response implies that the increased expression of CXCR3 may cause a stronger antiviral response in females (62). Amplified CXCR3 signaling due to XCI escape may thus contribute to increased immune activation and better ability to combat SARS-CoV-2 infection in women.

Toll-Like Receptor-7

Toll-like receptor-7 (TLR7)-mediated secretion of IFN-α has been demonstrated to play a key role in the response to coronavirus infections (13). The gene for TLR7 is located at Xp22.3 and participates in XCI escape, leading to overexpression in women (65). About 30% of immune cells from women and 19.3–39% of immune cells from XXY KS men display biallelic expression of TLR7 (66). In the presence of TLR7, CD27+ B cells were found to proliferate more quickly in female cells than non-KS cells, suggesting amplified TLR7 signaling in biallelic cells (66). When stimulated by TLR7, biallelic B cells were 2.4 times more likely to undergo IgG class switching compared to monoallelic B cells (66). Increased class switching suggests that women and KS males may have enhanced humoral immune response due to TLR7 overexpression. Estrogen levels likely contribute to the sex-based difference in TLR7 signaling, as immune cells from both men and women have been shown to increase TLR7 expression post-exposure to estradiol treatment (60, 67). Evidence suggests, however, that biallelic expression of TLR7 may also increase TLR7 signaling capacity. Transplanted pDCs from women produced a heightened TLR7-mediated IFN-α response to influenza virus and HIV pathogen-associated molecular pattern molecules (PAMPS), regardless of the sex of the mouse host (67). This finding implies a contributor to the IFN-α sex bias which is intrinsic to the immune cell. This factor is likely independent from hormone signaling, as similar increases in IFN-α were observed when comparing pDCs from men and women that were transplanted into female mice (67). As a result, the overexpression of TLR7 in biallelic pDCs may play a part in increasing secretion of antiviral IFN-α.

Further studies of 47, XXY karyotype KS—men with an extra X chromosome—and Turner's syndrome (TS)—women lacking an X chromosome with a 45, XO karyotype—implicate XCI escape in the sex-based difference in the immune response. KS men, but not TS women, typically undergo XCI. As a result, inclusion of genotypically diverse individuals may lend additional insights into the impact of XCI escape on TLR7 gene dosing. Lymphocytes purified from women and KS men expressed more copies of TLR7 mRNA when compared to men and TS women after exposure to TLR7 agonist CLO97 (65). As a result, XCI escape may be correlated with increased TLR7 signaling and more vigorous immune response to viral infections.

CXorf21

CXorf21 is located at gene locus Xp21.2 and escapes X-inactivation (68, 69). It is implicated in SLE, which predominantly affects women and KS males, and is expressed at higher rates in individuals with SLE (69). Lipopolysaccharide, IFN-γ, and IFN-α have been observed to increase CXorf21 expression in monocytes and B cells, suggesting possible roles in both innate and adaptive immunity (68). Specifically, CXorf21 may collaborate with TLR7 to maintain optimal lysosomal pH levels for degradation of pathogenic material, which APC display for T-cell recognition. TLR7 promotes CXorf21 expression, while CXorf21 decreases TLR7 transcription through a feedforward response. As a result, CXorf21 likely participates in the TLR7 pathway and may engage in the SARS-CoV-2 immune response. While its exact function is presently unknown, its overexpression in female APC suggests that CXorf21 may cooperate with TLR7 to contribute to the heightened antiviral response in women (68).

CD40L

The gene locus for CD40L has been identified as Xq26.3-27.1 (65). CD40L functions in several aspects of the adaptive immune response, including T-cell differentiation, immunoglobulin class switching, and formation of long-lived plasma cells and memory B cells (70). In particular, CD40L signaling acts on CD8 + T cells to mobilize the mucosal cytotoxic T lymphocyte (CTL) response to viral infection (71). A comparison of antigen presenting cells (APCs) from TS women, KS men, and individuals with typical karyotype found that cells from typical women expressed significantly more CD40L than those from typical men or TS women (65). Klinefelter Syndrome men yielded similar results to women possessing an XX karyotype, suggesting that XCI escape may confer an advantage against viral infections by increasing CD40L expression. Increased CD40L in individuals with an additional chromosome may cause greater T- and B-cell activation, leading to better ability to fight off viral infection.

Complement C4

Evidence suggests that the complement system may serve as the first line of defense against SARS-CoV infection. Mannose-binding lectin was found to be depleted in patients with SARS-CoV, suggesting that the complement system may aid in the response to coronavirus infection (72). The presence of mannose binding lectin similarly amplified the binding of complement C4 on SARS-CoV *in vitro*, supporting the activation of the lectin pathway in the SARS-CoV response. C4, which is located at the MHC, is of particular interest because of observed differences between sexes (22). C4 protein is more abundant in the cerebrospinal fluid and plasma of men compared to women, with a greater difference observed in men and women of childbearing age (20–50 years). Moreover, mutation in the C4 gene affects disease risk differently in men and women. Mutations which increase expression of C4 correlate with elevated risk of schizophrenia, SLE, and Sjogren's syndrome. Women have higher incidence rates of SLE and Sjogren's syndrome but lower incidence rates of schizophrenia, while the opposite is true for men. Moreover, the sex-based difference in disease incidence mirrors that of C4 protein levels and is most noticeable between men and women aged 20–50. As a result, the discrepancy in disease rates may be attributed to variable effects of C4 between men and women. The different actions of C4 in men and women may contribute to observed disparities in severity and incidence of SARS-CoV among sexes.

Human Leukocyte Antigen

Human leukocyte antigen (HLA) is located in the MHC gene group along with C4 and has been linked to AD, including type 1 diabetes and rheumatoid arthritis (22). Human leukocyte antigen enables differentiation between host cells and pathogens through

antigen presentation to the T-cell receptor (TCR). The HLA system shapes the TCR repertoire by bolstering or suppressing different T cell lines based on antigen exposure, and thus may affect the adaptive immune response to SARS-CoV-2.

Biological sex has been shown to affect HLA interaction with the TCR. In men with AD (MS and rheumatoid arthritis), CD8+ T cell clonal expansion is less dependent on HLA binding affinity when compared to women with AD, leading to greater production of T-cell clones with low TCR-MHC affinity in men (73). As MHC binding is required for effective T-cell activity, this finding is consistent with the observation that men are biased toward infections and non-reproductive system cancers (hypoactive T-cell response) while women are biased toward autoimmune disorders (hyperactive T-cell response). The sex-based difference in HLA signaling may thus contribute to sex-based differences in COVID-19.

NF-κB Essential Modulator

NF-κB essential modulator (NEMO), gene locus Xq28, serves as an activator of the NF-κB pathway (74). As the NF-κB pathway has been implicated in the response to SARS-CoV, NEMO may play a part in warding off SARS-CoV-2 infection (75). Evidence suggests that "one hit inactivation" may disproportionately place men at increased risk of complications from mutated NEMO due to the absence of cellular mosaicism (76). For instance, incontinentia pigmenti, caused by mutations in NEMO, causes lethality in men but has variable presentation in women. Skewed X-inactivation favoring the wildtype allele has been observed in heterozygous women, which supports the notion that X inactivation may confer a protective advantage against immunodeficiency disorders (77). Moreover, men with KS have been observed to escape lethality from incontinentia pigmenti (78). As a result, the presence of an additional X chromosome in women and KS men may decrease the likelihood of NEMO dysfunction and serve as an advantage in the SARS-CoV-2 response.

FoxP3

FoxP3 has been mapped to locus Xp11.23 (NCBI FOXP3). Mosaic expression of FoxP3 suggests decreased risk of non-functional FoxP3 in women, compared to men who possess one allele and experience "one hit inactivation" (58). Immune dysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX), caused by mutant FoxP3, primarily affects men. Because FoxP3 does not undergo skewed X inactivation, heterozygous women express both mutant and wildtype FoxP3 alleles equally (79). However, women heterozygous for mutant FoxP3 exhibit normal lymphocyte levels and normal immune response to infection. The participation of FoxP3 in positive feedback loops, in which FoxP3 protein further stimulates transcription of the FoxP3 gene, indicates that one functional copy of the gene may be sufficient to maintain appropriate levels of FoxP3 (80). As FoxP3 is critical to Treg-mediated immunosuppression, the protective effect of mosaicism implies an immunologic advantage for women at a population level (81). Low levels of Tregs have been associated with increased mortality rates in murine coronavirus-infected mice, while mice administered CD4+CD25+ Tregs yielded decreased mortality rates (40, 82). As a result, the ability to curb excessive activity by cytotoxic neutrophils, macrophages, and other immune cells may decrease risk of fatality from SARS-CoV and other coronaviruses (83). Although Tregs may offer benefit by reducing excessive tissue damage, they may also dampen the immune system and limit ability to clear an infection, indicating the need to strike a balance between the two. While Tregs may be less important in acute viral infections requiring an aggressive immune response, the disease characteristics for SARS-CoV-2 suggest that Tregs may play a crucial role in the antiviral response. Cellular mosaicism and the resulting improvement in genetic diversity may allow women to strike this balance more easily.

MicroRNA

MicroRNA (miRNA) are short, single stranded non-coding RNA that bind complementary sequences on target genes and block mRNA translation and degradation. Roughly 14% of all miRNA show a sex-biased expression pattern (84). There are 113 miRNA on the X chromosome and 2 miRNA on the Y chromosome (4). Many of these X-linked miRNAs target immuno-suppressive genes like FoxP3, CTLA4, CBL, and SOCS, preventing their translation or triggering their degradation. Given that women have two copies of the X chromosome, and that some of these genes may escape X-inactivation, this may help to explain the sex bias in immune responses. Additionally, it has been found that miRNA that are evolutionarily conserved are more often implicated in disease states (85), and male-specific miRNA evolve more quickly than female miRNA (84) and therefore are less conserved. Taken together, this suggests that female-specific miRNA have more pro-inflammatory effects than male-specific miRNA.

Several studies have shown the role of miRNA to control the host cell response to infections by RNA viruses and to control the virus' levels of infectivity via binding to viral RNA (86, 87). Following the binding, the miRNA can either inhibit translation and decrease viral infectivity or it can stabilize the RNA and effectively increase translation. These studies have shown miRNA interaction with RNA viruses such as Hepatitis C Virus (HCV), Human Immunodeficiency Virus (HIV), West Nile Virus (WNV), Dengue Virus (DV), and Influenza A Virus (IAV). In contrast, the viral RNA itself can have an impact on host miRNA to help evade the host immune system. This is exactly what was observed in the MERS and SARS-CoV infections. An *in silico* gene expression analysis (87) revealed that SARS-CoV upregulates miRNA-17, -574-5p, and -214 and MERS upregulates miRNA-628-5p, -6804-3p, -4289, -208a-3p, -510-3p, -18a-3p, -329-3p, -548-ax, -3934-5p, -4474-5p, -7974, -6868-5p, and -342-3p. Together, these contribute to viral evasion of the immune response. This data suggests that the novel SARS-CoV-2 virus may respond in a similar fashion and raise the possibility of miRNA as a therapeutic target.

Sex Hormones

Sex hormones are an important biological factor contributing to the gender-bias in the immune response, and can influence outcomes of disease severity in infections and autoimmunity (4,

88–90). Variations in sex hormone levels throughout the lifespan as with puberty, pregnancy, exogenous sex-hormone therapies, aging/menopause, and in transgender individuals modulate the immune response to pathogens therefore underlying the importance of their study. In general, estrogens are considered immuno-stimulatory and activate both the innate and adaptive immune responses and therefore women are able to clear pathogens more efficiently than men, whereas testosterone is immuno-suppressive, which may underlie the higher susceptibility and severity of infectious diseases in men (4). On the other hand, the stronger immune response in women is thought to underlie the disproportionately high prevalence of AD in women over men.

Sex hormones control both cellular and humoral components of the immune response and thus determine the sex-bias in susceptibility, manifestations and clinical outcomes in infections, AD and malignancies (4, 90). Immune cells bear estrogen receptors (ER) α and β, progesterone receptors (PR), and androgen receptors (AR), which are ligand-activated transcription factors. Sex hormones bind these receptors and trigger intracellular signaling cascades to regulate gene and protein expression to influence development, maturation, activation, and function of innate and adaptive immune cells during homoestasis and the immune response to infections. While the immune responses are in part mediated against the infectious agent and protective to the host, an overactive response such as overproduction of inflammatory cytokines can lead to severe immunopathology and organ damage and ultimately fatality, as is seen in certain respiratory viruses with complications of ARDS in the lungs during the SARS-CoV, MERS, and the current SARS-CoV-2 COVID-19 pandemic. Better understanding of the factors that control the immune response in a sex-specific manner is therefore crucial to not only understanding disease pathogenesis but also guiding treatment and prevention strategies and a first step toward personalized medicine.

The sex-biased factors that impact immunity have developmental origins beginning *in utero*, infancy and childhood (91). For example, placental hormones help shape fetal and neonatal immunity, and some of these influences are retained through adulthood. Estrogen and progesterone are important in alveolarization and surfactant production respectively. Sex hormones surge in a time of early infancy termed as "mini-puberty"; this influences the immune system and early childhood susceptibility to infections in boys versus girls. For some infections and AD, these differences in susceptibility and severity are retained through adulthood but may change or even reverse for some allergy-related conditions and AD.

Studies on the role of sex hormones in immune cells range from *ex vivo* cultures of human or mouse cells, or *in vivo* supplementation in mice after gonadectomy, including those assessing mice with genetic deletions of sex hormone receptors. Given the wide variations in human versus rodents *in vitro* versus *in vivo* systems, epidemiological studies have shown that there is not a universal paradigm regarding the role of gender or sex hormones on the immune response to respiratory viruses. It is hypothesized that the disease outcomes are ultimately a combination of the magnitude of the immune response and degree of host tissue damage (92, 93). There is a male bias when a weaker immune response contributes to damage, while a female bias may occur due to a stronger immune response that causes damage.

Estrogen and Innate Immunity

Estrogen-ER signaling regulates innate myeloid cells including pDCs, monocytes, neutrophils, and lymphoid cells, including innate lymphoid cells (ILC) (16). Estrogen is known to promote type I IFN synthesis, and female plasmacytoid DCs produce more type I IFN in response to viral nucleic acids and TLR-7 activation than males, which correlates with IRF 5 levels. Lower physiologic concentrations of estrogen are known to enhance the proinflammatory cytokines IL-12, IL-6, and IL-1β, while higher physiologic concentrations diminish their levels and in turn promote IL-10 regulatory cytokines (16). Furthermore, estradiol promotes the differentiation of murine BM-derived DCs by increasing IRF4 transcription factor levels. Estrogens contribute to delayed neutrophil apoptosis and can modulate chemotaxis and NO production *in vitro*. The lung-resident alveolar macrophages are important in respiratory infections and produce type I IFN for viral clearance. Although they express both ERα and AR, sex differences or the role of sex hormones on these cells during respiratory viral infections have not been reported (16). Differentiation of M1 and M2 subtypes by a type 1 IFN-driven or a type 2 IL-4/IL-13-driven response are known to be influenced in allergic asthma, where females/ERα promotes the M2 phenotype important for tissue repair. These findings imply that wherein estrogen and ERa enhance while AR may dampen the type 2 responses important for lung tissue repair post-viral infections. ILC2s important in tissue repair and secrete IL-5/IL-13 are the prominent subtype in murine lungs and their numbers are increased in female mice and in humans compared to males. While these cells predominantly express AR, there is tissue specific regulation by sex hormones and estrogen-ER signaling promoted uterine over lung ILC2. Elevated numbers in IAV infections may provide superior tissue repair, however their plasticity to convert to ILC-1 like cells and IFN-g production may make them more pathogenic and contribute to immunopathology (16).

Estrogen and Adaptive Immunity

In general, estrogens are immune-stimulatory and are known to be involved in T-cell development, activation, differentiation and function (4). Estrogen-ER signaling was shown to be necessary for normal thymic size and development, and furthermore estrogen is known to promote extrathymic T-cell differentiation in the liver. Its role in T-cell homeostasis with respect to cell survival and proliferation is complex and varies depending on cell type, context, and concentration, where physiologic doses of estradiol suppress apoptosis whereas pharmacologic doses suppress proliferation in cancer cells. Estrogen and ERα promote CD4 T-cell activation and deletion of ERα led to altered transcriptomics with reduced levels of genes involved in T-cell activation. Estrogen controls cell metabolism and genes involved

in metabolic activity important to stimulate T-cell differentiation and stimulate mitochondrial function.

Estrogen increases signaling through NK-κB to activate production of inflammatory cytokines including IL-1β, IL-10, and IFN-γ in mouse splenocytes (4). Estrogen is known to suppress IL-2 cytokine production in human T cells (94) and rat splenocytes. Accordingly, lower IL-2 levels are observed during the luteal phase of the menstrual cycle in healthy young women and thought to contribute to the observed increase in pre-menstrual infections. Estrogen is known to increase proliferation of CD4 T cells and increase CD4 Th1 differentiation and IFN-γ cytokine production which are necessary in the cell mediated response to viral infections and in addition increases the inflammatory response mediated by IFN-γ via activation of iNOS, NO, and COX2. CD4 T follicular helper (Tfh) cells are crucial for providing cognate help to B cells and promote class switching and somatic hypermutation to produce antibodies. Estrogen was shown to promote the expression of Calcineurin and CD40L in human T cells (95), molecules important for help to B cells in the antibody response. T cells traffic within the body to peripheral tissue sites of infection and migrate across chemokine gradients via chemokine receptors expressed on their surface. Estrogen promotes both chemokines as well as chemokine receptor expression as evidenced by *ex vivo* and *in vivo* studies in mice (4). Female mice expressed higher levels of chemokine receptors CCR1-CCR5 in response to chemokines and mice administered estrogen expressed increased levels of MCP-1, MCP-5, Eotaxin, and SDF. Estrogens also enhance CD8 T-cell activity and suppress Th17 immune responses.

The role of Tregs in response to viral infections is complex. Estrogen increases FoxP3 levels and Tregs *in vitro* and correlations have been observed *in vivo*. Increased numbers of circulating Treg cells are observed during the late follicular phase of the menstrual cycle in fertile non-pregnant women which dropped in the luteal phase, correlating with β-estradiol levels. In women with recurrent spontaneous abortions (RSA), lower Treg levels were found in both follicular and luteal phases and in postmenopausal women. The suppressive capacity of these Tregs was also lower in case of RSA.

Estrogen promotes B-cell homeostasis, activation, maturation, and differentiation and enhances immunoglobulin production (4). These properties make women and female mice able to mount greater magnitudes of neutralizing antibody responses to infections and thus contribute to protection against respiratory viral infections including the SARS-CoV infections. Estrogen administration led to increased marginal zone B cells in the spleen and in transgenic mice led to elevated anti-dsDNA antibodies. Estrogen promoted the expansion of high-affinity antibody-producing B cells and also promoted survival by increasing expression of the Bcl-2 anti-apoptotic molecule (96). In addition, higher levels of B lymphocyte stimulator (Blys) also called B cell activating factor (BAFF) were observed in female C57Bl/6 mice. Estrogen administration elevated BAFF levels and this was reverted in mice deficient in ERα, STAT1, or IRF5 (97). Besides controlling B-cell activation which enhances the Ig antibody response, ER can directly control the Ig as ER-binding ERE have been found within the heavy chain locus of Ig by an ERα antibody-mediated ChIP-sequencing analysis of genomic DNA (4).

Progesterone and Androgens

Immune cells express PR and AR, and progesterone, androgen, and testosterone in particular are considered immunosuppressive and may counteract the effects of estrogens, contributing to the observed increased susceptibility to the SARS-CoV-2 and disease in men (98–100). Progesterone downregulates IL-1β and TNF proinflammatory cytokine production by BM-DCs in mice, and testosterone is known to decrease cytokines including IFN-γ and TNF and increase regulatory cytokines such as IL-10. Androgen receptor-deficient mice exhibit reduced numbers of neutrophils and accordingly increased susceptibility of male mice to SARS-CoV infection correlated with accumulation of neutrophils in the lung. Progesterone reduces T-cell proliferation and T-cell-dependent antibody responses in human peripheral blood and cell line or mouse studies. Furthermore, progesterone regulates CD4 Th differentiation and cytokine production with increased IL-4, increased Treg cell differentiation, and reduced IFN-γ, Th17 responses. In cytotoxic CD8 T cells, progesterone reduces IFN-γ and cytotoxicity. Its effects on B cells included reduced class switch recombination and reduced T cell dependent antibody production. Normal testosterone levels are associated with normal respiratory capacity, whereas plasma testosterone levels decline in men with increasing age with observed associations between an increase of pro-inflammatory states and decline in testosterone in aging men. Furthermore, hypogonadism is associated with elevated pro-inflammatory cytokines and testosterone decreases levels of IL-1β, IL-6, and TNF-α (98). On the other hand, high androgen levels may promote or contribute to infection because AR mediated transcription of TMPRSS2 protease which is important for viral entry into host cells.

A proposed androgen sensitivity model provides a link between increased disease severity in men and the role of androgens in COVID-19 (101). Androgen sensitivity is primarily determined by genetic variants of the AR. Specifically, shorter CAG repeat polymorphisms in the AR gene have been associated with both androgen sensitivity and more severe COVID-19 symptoms. Shorter CAG repeat polymorphisms may cause overexpression of the transmembrane protease, serine 2 (TMPRSS2) gene due to greater activation of the AR. TMPRSS2 overexpression in turn may enable greater viral entry and replication through cleavage of both the SARS-CoV-2 spike protein and ACE2 receptor, facilitating viral uptake and virus-cell fusion (Wambier). In addition, it has been observed that SARS-CoV cleavage by TMPRSS2 releases spike protein fragments that act as decoys for neutralizing antibodies (102). A similar mechanism may function in SARS-CoV-2 infection to dampen humoral immunity. As TMPRSS2 transcription is activated upon AR binding, elevated TMPRSS2 in men and individuals with high androgen levels may contribute to sex-based disparities in COVID-19 severity through the following mechanisms: viral replication promotion and humoral immunity suppression.

While men are generally predisposed to these effects due to higher testosterone levels compared to women, individuals

with hyperandrogenism and other conditions may similarly be impacted. For example, women with hirsutism and polycystic ovarian syndrome (PCOS), as well as women taking progesterone-based birth control, may be at greater risk for more severe COVID-19 symptoms (101). Markers of androgen sensitivity such as PCOS, androgenetic alopecia, and prostatic hyperplasia may thus be used as clinical signs of vulnerability. The high rates of androgenetic alopecia observed in hospitalized COVID-19 patients supports the theory that elevated androgens may contribute to more severe COVID-19 symptoms by increasing TMPRSS2 expression in target tissues.

Clinical Trials With Estrogen and Progesterone for COVID-19

In this pandemic, men are more acutely ill and exhibit higher death rates with disproportionately higher numbers in ICU and requiring ventilators compared to women. Even pregnant women had lower rates and less severe disease and complications than men. Estrogen and progesterone levels rise exponentially during pregnancy, causing a shift in the immune response, and this may underlie the observed protective effects. The importance of these female sex hormones in the immune response to infections has triggered two clinical trials with sex hormone administration to patients with COVID-19. One trial at the Renaissance School of Medicine Stony Brook University, Long Island, New York, is administering Estrogen to 110 adult patients, including men and women over 55, who present to the ER with symptoms of COVID-19 like fever, cough, shortness of breath or pneumonia. Half will receive a single-use transdermal estradiol patch for 7 days, and the other half will serve as a control group and receive standard of care. The second, smaller randomized controlled trial with 40 male patients at Cedars-Sinai hospital in Los Angeles will administer progesterone in an effort to suppress the overactive immune response and mitigate immunopathology. Inpatients with mild to moderate disease will be included and half will receive progesterone 100 mg subcutaneous twice daily for 5 days, and the other half is a control group. Since progesterone is immune-suppressive and diminishes the proinflammatory response, this trial is intended to determine whether progesterone treatment can reduce the incidence of cytokine storm and related immunopathology leading to ARDS.

Microbiome

It is well-known that the commensal bacteria in the GI tract impact the immune response. Some possible mechanisms involve microbiota affecting and regulating cytokine production (103), while others involve microbiota modulation of the production of mucous and antiviral defensins and ROS (104). In regard to viral infections, however, some microbiota elicit protective effects, while others serve as a route of viral entry and infection. For example, the Lactobacillus genus prevents murine norovirus replication *in vitro*, and there is *in vivo* evidence that this genus is decreased in a mouse that is affected by norovirus. In response to Influenza and WNV, gut microbiota secrete IgA and upregulate TLR-7 in the respiratory mucosa (105) in order to promote activation of important components of antiviral immunity—cytotoxic T lymphocytes, Th1 cells, and inflammasomes. On the other hand, human and murine norovirus utilize the commensal gut bacteria as a place to harbor and/or break through the mucosa—leading to infection.

There is currently less known about the interaction between SARS-CoV-2 and gut commensal bacteria, but it has been noted that COVID-19 patient fecal samples contain higher numbers of the Prevotella genus (106). It is therefore possible that there is an interaction between these bacteria and the virus, though the exact relationship is unknown. One possible mechanism is that, similarly to the human norovirus, the normally commensal bacteria are harboring the virus and are producing a cytokine response that is inappropriate for the response to the infection. This ultimately would result in dysbiosis and a worse outcome for the patient. Alternatively, a relationship between ACE-2 and the gut microbiome may play a role in the immune response. SARS-CoV and SARS-CoV-2 bind to the ACE-2 receptor and cause downregulation. This protein is critical for the transportation of tryptophan across the epithelium, which then normally increases the production of antimicrobial peptides that affect the composition of the microbiome (107). Lack of this peptide production would likely result in dysbiosis and an impaired immune response.

Recently, it has been noted that sex hormones have a large effect on the microbiome. Particularly, higher levels of systemic estrogen, like those seen in women, are positively associated with the richness and diversity of the fecal microbiome (108). Additionally, germ-free female mice have higher baseline antibody levels than germ-free male mice (109). Women have higher expression of antiviral and pro-inflammatory genes (92), and male mice show higher levels of the anti-inflammatory molecules TGF-β, IL-10, and FoxP3. Additionally, male mice who received an *in vivo* transplant of female microbiota showed increased T cell precursors in the thymus and decreased levels of RORγt and FoxP3 + cells (110). RORγt cells are important for controlling the Th17 cell response, while FoxP3 + cells are Tregs that help to control the immune response. Taken together, these studies suggest that female sex hormones, particularly estrogen, have a pro-inflammatory effect (4) that promotes a more robust response to infection.

In addition to lacking the stimulatory effects of estrogen, men also produce androgens that seem to have a protective mechanism against the immune response. Testosterone is known to decrease the ability of NK cells to produce and secrete IFN-γ, an important mediator of the immune response (111). Men also have lower CD3+, CD4+ counts, lower CD4+/CD8+ ratios, and lower Th1 responses than women (92). Furthermore, the testosterone surge at puberty in male mice dampens B- and T-cell development (112).

Commensal bacteria in the gastrointestinal tract have a role in regulation of testosterone levels (109). An *in vivo* study found that the number of species in microbiomes of mice was not significantly different during the pre-pubescent stage but was significantly different following puberty (113). This suggests that hormone changes during puberty drive changes in the microbiome. Further, the microbiome elevates androgens to a level that confers protection from type 1 diabetes in mice (112, 113), which illustrates the synergistic effect of the male hormones

and the microbiome. While this is thought to be a major factor in the protection against autoimmunity, it is also reasonable to think that the immune response may be dampened below the level that is needed for a strong response to pathogens. Overall, these findings suggest that the microbiome is an important biological factor in the sex-bias in response to infection and may be involved in the SARS-CoV-2 responses.

ACE2

The SARS-CoV-2 spike (S) glycoprotein enters the host cell via binding to the ACE2 receptor. This binding leads to the subsequent downregulation of ACE2, which is considered to be protective against lung injury. ACE2 downregulation leads to pathway shunting toward ACE enzyme and a buildup of angiotensin II. Stimulation of AGTR1a receptor by angiotensin II leads to endothelial cell permeability which may explain the increase in pulmonary pathology with decreasing levels of expressed ACE2.

The location of ACE2 on the X chromosome suggests possible genetic influence in the elevated male mortality rate. As the binding affinity between SARS-CoV-2S protein and ACE2 dictates transmissibility and disease severity, the presence of two cell lines and subsequently two ACE2 variants in women may contribute to the sex-based differences observed in COVID-19 patients. For example, mutations in ACE2 in one cell line may alter the catalytic site and lead to divergent viral susceptibilities between cell populations decreasing peak viral load (114). Notable protein-protein interactions between ACE2 and SARS-CoV-2 include a salt bridge formation between Lys317 of the SARS-CoV-2 receptor binding domain (RBD) and Asp30 of ACE2 as well as a Van der Waals interaction between Leu472 of the RBD and Met82 of ACE2 (115). Substitutions in these amino acids, among other mutations, may alter binding affinity between the RBD and receptor, limiting the ability of the virus to enter the cell and propagate.

Variable expression of ACE2 may also influence patient outcomes. Viral entry into a cell causes downregulation of ACE2, which may be detrimental in patients already deficient in ACE2 (116). ACE2 downregulation leads to pulmonary edema, alveolus hyalinization, and leukocyte accumulation. Cecal ligation and perforation of ACE2 knockout mice has also been shown to increase lung failure and tissue damage, indicating that ACE2 may confer a protective role in microbial infection (117). Gene dosage, however, likely does not contribute to the sex-based difference in SARS-CoV-2 response, as evidence suggests that sexual dimorphism in ACE2 expression persists in renal tissue but not cardiac or pulmonary tissue under non-pathological conditions (118). Moreover, changes in chromosome dosage was not observed to affect ACE2 expression in mice that had undergone gonadorectomy. This finding implies possible hormonal but not chromosomal effects in ACE2 expression levels.

Despite limited evidence supporting the effect of chromosome dosage in the increased male mortality rate, cellular mosaicism in women may offer protection against immune deficiency. As males are hemizygous for ACE2, they experience "one hit inactivation" due to the absence of the "back-up" allele present in women. As a result, the effect of detrimental mutations to ACE2 may be more pronounced in men than women, altering the clinical course in male versus female patient populations.

The ACE2 receptor is expressed in the type II pneumocytes of the lungs and also in other tissues, including the heart, tubular epithelial cells in kidneys, testis, adipose tissue, and the enterocytes in the gastrointestinal tract and vascular endothelial cells (119). A recent study evaluated ACE2 expression in older men and women with heart failure and found in two independent cohorts that circulating plasma concentrations of ACE2 were higher in men than in women (120). This may reflect differences in tissues from men versus women. Two studies utilized systems biology approaches of meta-analysis, co-expression and network analysis to draw information on the expression, regulation and gender bias of ACE2 receptor expression. One study (121) evaluated datasets from the Genotype-Tissue Expression (GTEx) project, the cancer genome atlas (TCGA) program and the human protein atlas (HPA) database to evaluate ACE2 expression in 31 normal human tissues, comparing men versus women and younger (ages = 49 years) versus older (ages = 49 years) individuals and further correlating ACE2 expression with immune signature enrichment (CD8+ T cells, IFN response, B cells, and NK cells) across tissues. The highest levels of ACE2 expression were found in the small intestine, testis, kidneys, heart, thyroid, and adipose tissue, medium levels in the lungs, colon, liver, bladder, and adrenal gland, and lowest in blood, spleen, bone marrow, brain, blood vessels, and muscle. While they did not find a significant difference in gene expression between men and women, ACE2 expression in the lungs was positively correlated with immune signatures in men and negatively in women. In addition the HPA database showed high levels of both ACE2 gene and protein in the gastrointestinal tract (duodenum, small intestine, colon, and rectum), kidney, gallbladder, and male tissues (testis and seminal vesicle). Taken together these data suggest that the differential host immune responses may underlie the gender-bias of the remarkably distinct clinical outcomes. The other study of patients with severe COVID-19 who had comorbidities (122) evaluated data from over 700 lung transcriptome samples and found that ACE2 was highly expressed in these patients, compared to controls. Correlation and network analyses revealed many histone-related genes HAT1, HDAC2, and KDM5B. Suggesting epigenetic regulation of ACE2 in the human lung.

ACE2 is known to be expressed in Leydig cells of both mice and humans, albeit testosterone-independent, and is thought to contribute to steroid synthesis (123, 124). It is also expressed in ovarian granulosa cells and its levels increase in correlation with increasing LH levels. In addition to expression in the gonads, ACE2 expression and activity is influenced by sex hormones in adipose tissue, myocardium, and kidneys. ACE and ACE2 are intricately involved in the renin angiotensin system for fluid/electrolyte balance and cardiovascular homeostasis and control of metabolic factors contributing to obesity, hypertension and related cardiovascular complications. One study examined the role of sex hormones on cardiac ACE and ACE2 activity. Higher ACE and ACE2 activity and cardiac hypertrophy was found in male rats compared to female rats which was

reduced after orchiectomy, while ovariectomy elevated ACE2 and hypertrophy in females (125). In male mice, chronic high fat diet (HFD) led to decreased ACE2 expression in the kidneys. In female mice, HFD increased adipose tissue ACE2 which was reversed by ovariectomy implying that estrogen increases ACE2 expression and activity in adipose tissue and kidneys. Furthermore global deficiency of the ACE2 gene increased HFD-induced obesity hypertension in male mice (126, 127). Importantly, ovariectomy or treatment with an ER antagonist in SARS-CoV infected female mice increased the mortality rate therefore, suggesting a protective effect for the ER signaling pathway in mice (3).

While sex hormones influence ACE2 expression and activity to influence outcomes in obesity, hypertension, and related comorbidities, thus influencing COVID-19 outcomes, the effect of COVID-19 on male sex hormones has been recently explored. Given that the ACE2 receptor is expressed in the testes, a study from Hubei province of China reports that the COVID-19 impacts male gonadal function and observed alterations in hormone levels. They studied 81 men with COVID-19 and found that serum luteinizing hormone (LH) levels were increased while the ratio of testosterone to LH and the ratio of follicle stimulating hormone (FSH) to LH were significantly lower compared with 100 age-matched healthy men (128). Recent reports of increased frequency of venous thromboembolism, associated with worse outcomes in patients with COVID-19 warrant caution in treatment with testosterone, specifically in hypogonadal men with greater genetic predisposition.

Immune Response to Vaccines

Besides its role in infection immunity, sex is equally important in the immune response to vaccines (129, 130). Women not only mount stronger antibody and T-cell responses to vaccinations than men, but also suffer more adverse events. Yet there is a serious lack of attention to gender in vaccine trials. This leads to inappropriate dosage of vaccines as evidenced by the fact that the same magnitude of protective immunity is achieved by half the dose of seasonal influenza vaccine in women compared to men. Likewise, these gender-blind vaccination strategies lead to increased adverse effects in women. Increased hospitalizations and mortality have been observed in female infants and girls following DPT, measles and oral polio vaccinations (130). Sex-based biological factors include differences across the immune system within innate immunity, antibody responses and T cell responses. Genetics, sex hormones, epigenetic factors, nutrition, and the microbiome are important biological contributors to these sex-based differences. Vaccine-related research and clinical trials, including those currently underway for COVID-19, must thus include sex as a key variable when measuring and reporting outcomes of immunogenicity and reactogenicity. This information would help tailor vaccine dosage and strategies appropriately to maximize protective immunity while minimizing adverse effects.

CONCLUSION

The COVID-19 pandemic has revealed a striking gender-bias with increased case and mortality rates in men compared with women across the lifespan. Besides behavioral and lifestyle factors, sex-based physiological differences influence the host immune response to infections. Sex chromosome linked genes, sex hormones, and the microbiome control aspects of the innate and adaptive immune responses to infection. Genetics and sex hormones also regulate and influence aspects of viral entry including expression and activity of ACE2 and TMPRSS2. These differences not only affect the risk/susceptibility to infection but also the disease course/clinical outcomes and response/adverse effects to vaccines. Better understanding of these factors is necessary to tailor therapies and vaccine strategies in a step toward sex-based personalized medicine.

AUTHOR CONTRIBUTIONS

VM conceptualized the article. All authors contributed to the literature review, writing, and finalizing the manuscript.

FUNDING

VM is funded through an NIH NIAMS grant (R01 AR076894).

ACKNOWLEDGMENTS

We thank Dr. Robert Shmerling for critical review of the manuscript and helpful suggestions.

REFERENCES

1. Shi Y, Wang Y, Shao C, Huang J, Gan J, Huang X, et al. COVID-19 infection: the perspectives on immune responses. *Cell Death Differ.* (2020) 27:1451–4. doi: 10.1038/s41418-020-0530-3
2. Guan W, Ni Z, Hu Y, Liang W, Ou C, He J, et al. Clinical characteristics of coronavirus disease 2019 in China. *New Engl J Med.* (2020) 382:1708–20. doi: 10.1056/NEJMoa2002032
3. Channappanavar R, Fett C, Mack M, Eyck PPT, Meyerholz DK, Perlman S. Sex-based differences in susceptibility to severe acute respiratory syndrome coronavirus infection. *J Immunol.* (2017) 198:4046–53. doi: 10.4049/jimmunol.1601896
4. Moulton VR. Sex hormones in acquired immunity and autoimmune disease. *Front Immunol.* (2018) 9:2279. doi: 10.3389/fimmu.2018.02279
5. Muus C, Luecken MD, Eraslan G, Waghray A, Heimberg G, Sikkema L, et al. Integrated analyses of single-cell atlases reveal age, gender, and smoking status associations with cell type-specific expression of mediators of SARS-CoV-2 viral entry and highlights inflammatory programs in putative target cells. *BioRxiv.* (2020). [Preprint] doi: 10.1101/2020.04.19.049254
6. Zhang H, Penninger JM, Li Y, Zhong N, Slutsky AS. Angiotensin-converting enzyme 2 (ACE2) as a SARS-CoV-2 receptor: molecular mechanisms and potential therapeutic target. *Intensive Care Med.* (2020) 46:586–90. doi: 10.1007/s00134-020-05985-9

7. David R. Are men and women different? *Nat Rev Immunol.* (2009) 9:605–605. doi: 10.1038/nri2631
8. Li S-W, Wang C-Y, Jou Y-J, Huang S-H, Hsiao L-H, Wan L, et al. SARS Coronavirus papain-like protease Inhibits the TLR7 signaling pathway through removing Lys63-linked polyubiquitination of TRAF3 and TRAF6. *Int J Mol Sci.* (2016) 17:678. doi: 10.3390/ijms17050678
9. Souyris M, Mejía JE, Chaumeil J, Guéry J-C. Female predisposition to TLR7-driven autoimmunity: gene dosage and the escape from X chromosome inactivation. *Semin Immunopathol.* (2019) 41:153–64. doi: 10.1007/s00281-018-0712-y
10. Channappanavar R, Fehr AR, Vijay R, Mack M, Zhao J, Meyerholz DK, et al. Dysregulated Type I Interferon and Inflammatory monocyte-macrophage responses cause lethal pneumonia in SARS-CoV-infected mice. *Cell Host Microbe.* (2016) 19:181–93. doi: 10.1016/j.chom.2016.01.007
11. Channappanavar R, Fett C, Mack M, Ten Eyck PP, Meyerholz DK, Perlman S. Sex-based differences in susceptibility to SARS-CoV infection. *J Immunol.* (2017) 198:4046–53. doi: 10.4049/jimmunol.1601896
12. Channappanavar R, Fehr AR, Zheng J, Wohlford-Lenane C, Abrahante JE, Mack M, et al. Response timing relative to virus replication determines MERS coronavirus infection outcomes. *J Clin Invest.* (2019) 129:3625–39. doi: 10.1172/JCI126363
13. Cervantes-Barragan L, Züst R, Weber F, Spiegel M, Lang KS, Akira S, et al. Control of coronavirus infection through plasmacytoid dendritic-cell-derived type I interferon. *Blood.* (2007) 109:1131–7. doi: 10.1182/blood-2006-05-023770
14. Haick AK, Rzepka JP, Brandon E, Balemba OB, Miura TA. Neutrophils are needed for an effective immune response against pulmonary rat coronavirus infection, but also contribute to pathology. *J Gen Virol.* (2014) 95:578–90. doi: 10.1099/vir.0.061986-0
15. Sawant KV, Poluri KM, Dutta AK, Sepuru KM, Troshkina A, Garofalo RP, et al. Chemokine CXCL1 mediated neutrophil recruitment: role of glycosaminoglycan interactions. *Sci Rep.* (2016) 6:1–8. doi: 10.1038/srep33123
16. Kadel S, Kovats S. Sex hormones regulate innate immune cells and promote sex differences in respiratory virus infection. *Front Immunol.* (2018) 9:1653. doi: 10.3389/fimmu.2018.01653
17. Geering B, Simon H-U. Peculiarities of cell death mechanisms in neutrophils. *Cell Death Differ.* (2011) 18:1457–69. doi: 10.1038/cdd.2011.75
18. Rosenberg HF, Dyer KD, Domachowske JB. Respiratory viruses and eosinophils: exploring the connections. *Antiviral Res.* (2009) 83:1–9. doi: 10.1016/j.antiviral.2009.04.005
19. Sanchez-Legrand F, Smith TF. Interaction of paramyxoviruses with human basophils and their effect on histamine release. *J Allergy Clin Immunol.* (1989) 84:538–46. doi: 10.1016/0091-6749(89)90368-0
20. Jiang Y, Zhao G, Song N, Li P, Chen Y, Guo Y, et al. Blockade of the C5a–C5aR axis alleviates lung damage in hDPP4-transgenic mice infected with MERS-CoV. *Emerg Microbes Infect.* (2018) 7:1–12. doi: 10.1038/s41426-018-0063-8
21. Mackey E, Ayyadurai S, Pohl CS, D' Costa S, Li Y, Moeser AJ. Sexual dimorphism in the mast cell transcriptome and the pathophysiological responses to immunological and psychological stress. *Biol Sex Differ.* (2016) 7:60. doi: 10.1186/s13293-016-0113-7
22. Kamitaki N, Sekar A, Handsaker RE, Rivera H, De Tooley K, Morris DL, et al. Complement genes contribute sex-biased vulnerability in diverse disorders. *Nature.* (2020) 20:1–7. doi: 10.1038/s41586-020-2277-x
23. Gralinski LE, Sheahan TP, Morrison TE, Menachery VD, Jensen K, Leist SR, et al. Complement activation contributes to severe acute respiratory syndrome coronavirus pathogenesis. *MBio.* (2018) 9:e01753-18. doi: 10.1128/mBio.01753-18
24. Gaya da Costa M, Poppelaars F, van Kooten C, Mollnes TE, Tedesco F, Würzner R, et al. Age and sex-associated changes of complement activity and complement levels in a healthy caucasian population. *Front Immunol.* (2018) 9:2664. doi: 10.3389/fimmu.2018.02664
25. Seth RB, Sun L, Chen ZJ. Antiviral innate immunity pathways. *Cell Res.* (2006) 16:141–7. doi: 10.1038/sj.cr.7310019
26. Savarin C, Bergmann CC. Fine tuning the cytokine storm by IFN and IL-10 following neurotropic coronavirus encephalomyelitis. *Front Immunol.* (2018) 9:3022. doi: 10.3389/fimmu.2018.03022
27. Zheng M, Gao Y, Wang G, Song G, Liu S, Sun D, et al. Functional exhaustion of antiviral lymphocytes in COVID-19 patients. *Cell Mol Immunol.* (2020) 17:533–5. doi: 10.1038/s41423-020-0402-2
28. Lutz CT, Al-Attar A, Presnell SR, Peterson CA, Thomas DT. The effect of sex on immune cells in healthy aging: elderly women have more robust natural killer (NK) lymphocytes than do elderly men. *J Immunol.* (2016) 196:2–204.
29. Hao S, Zhao J, Zhou J, Zhao S, Hu Y, Hou Y. Modulation of 17beta-estradiol on the number and cytotoxicity of NK cells in vivo related to MCM and activating receptors. *Int Immunopharmacol.* (2007) 7:1765–75. doi: 10.1016/j.intimp.2007.09.017
30. Wong CK, Lam CWK, Wu AKL, Ip WK, Lee NLS, Chan IHS, et al. Plasma inflammatory cytokines and chemokines in severe acute respiratory syndrome. *Clin Exp Immunol.* (2004) 136:95–103. doi: 10.1111/j.1365-2249.2004.02415.x
31. Dashraath P, Wong JLJ, Lim MXK, Lim LM, Li S, Biswas A, et al. Coronavirus disease 2019 (COVID-19) pandemic and pregnancy. *Am J Obstet Gynecol.* (2020) 222:521–31. doi: 10.1016/j.ajog.2020.03.021
32. Zhou Y, Fu B, Zheng X, Wang D, Zhao C, Qi Y, et al. Pathogenic T cells and inflammatory monocytes incite inflammatory storm in severe COVID-19 patients. *Natl Sci Rev.* (2020) 7:998–1002. doi: 10.1093/nsr/nwaa041
33. Klein SL. Immune cells have sex and so should journal articles. *Endocrinology.* (2012) 153:2544–50. doi: 10.1210/en.2011-2120
34. Khalil A, Kalafat E, Benlioglu C, O'Brien P, Morris E, Draycott T, et al. SARS-CoV-2 infection in pregnancy: A systematic review and meta-analysis of clinical features and pregnancy outcomes. *EClinicalMedicine.* (2020) 2020:100446. doi: 10.1016/j.eclinm.2020.100446
35. Ellington S. Characteristics of women of reproductive age with laboratory-confirmed sars-cov-2 infection by pregnancy status — United States, January 22–June 7, 2020. *MMWR Morb Mortal Wkly Rep.* (2020) 69:769–75. doi: 10.15585/mmwr.mm6925a1
36. Pacha O, Sallman MA, Evans SE. COVID-19: a case for inhibiting IL-17? *Nat Rev Immunol.* (2020) 20:1–2. doi: 10.1038/s41577-020-0328-z
37. Wu D, Yang XO. TH17 responses in cytokine storm of COVID-19: An emerging target of JAK2 inhibitor Fedratinib. *J Microbiol Immunol Infect.* (2020) 53:368–70. doi: 10.1016/j.jmii.2020.03.005
38. Voskuhl R. Sex differences in autoimmune diseases. *Biol Sex Differ.* (2011) 2:1. doi: 10.1186/2042-6410-2-1
39. Jadidi-Niaragh F, Mirshafiey A. Th17 cell, the new player of neuroinflammatory process in multiple sclerosis. *Scand J Immunol.* (2011) 74:1–13. doi: 10.1111/j.1365-3083.2011.02536.x
40. Anghelina D, Zhao J, Trandem K, Perlman S. Role of regulatory T cells in coronavirus-induced acute encephalitis. *Virology.* (2009) 385:358–67. doi: 10.1016/j.virol.2008.12.014
41. Li Z, Li D, Tsun A, Li B. FOXP3+ regulatory T cells and their functional regulation. *Cell Mol Immunol.* (2015) 12:558–65. doi: 10.1038/cmi.2015.10
42. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor foxp3. *Science.* (2003) 299:1057–61. doi: 10.1126/science.1079490
43. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol.* (2003) 4:330–6. doi: 10.1038/ni904
44. Moon C. Fighting COVID-19 exhausts T cells. *Nat Rev Immunol.* (2020) 20:277–277. doi: 10.1038/s41577-020-0304-7
45. Bekeredjian-Ding IB, Berkeredjian-Ding IB, Wagner M, Hornung V, Giese T, Schnurr M, et al. Plasmacytoid dendritic cells control TLR7 sensitivity of naive B cells via type I IFN. *J Immunol.* (2005) 174:4043–50. doi: 10.4049/jimmunol.174.7.4043
46. Bekeredjian-Ding I, Jego G. Toll-like receptors–sentries in the B-cell response. *Immunology.* (2009) 128:311–23. doi: 10.1111/j.1365-2567.2009.03173.x
47. Quinti I, Lougaris V, Milito C, Cinetto F, Pecoraro A, Mezzaroma I, et al. A possible role for B cells in COVID-19? Lesson from patients with agammaglobulinemia. *J Allergy Clin Immunol.* (2020) 146:13. doi: 10.1016/j.jaci.2020.04.013

48. Channappanavar R, Zhao J, Perlman S. T cell-mediated immune response to respiratory coronaviruses. *Immunol Res.* (2014) 59:118–28. doi: 10.1007/s12026-014-8534-z
49. Ng O-W, Chia A, Tan AT, Jadi RS, Leong HN, Bertoletti A, et al. Memory T cell responses targeting the SARS coronavirus persist up to 11 years post-infection. *Vaccine.* (2016) 34:2008–14. doi: 10.1016/j.vaccine.2016.02.063
50. Channappanavar R, Fett C, Zhao J, Meyerholz DK, Perlman S. Virus-specific memory CD8 T cells provide substantial protection from lethal severe acute respiratory syndrome coronavirus infection. *J Virol.* (2014) 88:11034–44. doi: 10.1128/JVI.01505-14
51. Yang L-T, Peng H, Zhu Z-L, Li G, Huang Z-T, Zhao Z-X, et al. Long-lived effector/central memory T-cell responses to severe acute respiratory syndrome coronavirus (SARS-CoV) S antigen in recovered SARS patients. *Clin Immunol.* (2006) 120:171–8. doi: 10.1016/j.clim.2006.05.002
52. Hewagama A, Patel D, Yarlagadda S, Strickland FM, Richardson BC. Stronger inflammatory/cytotoxic T-cell response in women identified by microarray analysis. *Genes Immun.* (2009) 10:509–16. doi: 10.1038/gene.2009.12
53. Ahmed SF, Quadeer AA, McKay MR. Preliminary identification of potential vaccine targets for the COVID-19 Coronavirus (SARS-CoV-2) based on SARS-CoV immunological studies. *Viruses.* (2020) 12:254.
54. Long Q-X, Liu B-Z, Deng H-J, Wu G-C, Deng K, Chen Y-K, et al. Antibody responses to SARS-CoV-2 in patients with COVID-19. *Nat Med.* (2020) 26:845–8. doi: 10.1038/s41591-020-0897-1
55. Sethuraman N, Jeremiah SS, Ryo A. Interpreting Diagnostic Tests for SARS-CoV-2. *JAMA.* (2020) 323:2249–51. doi: 10.1001/jama.2020.8259
56. Avery DT, Bryant VL, , De Malefyt RW, Tangye SG. IL-21-Induced isotype switching to IgG and IgA by human naive B cells is differentially regulated by IL-4. *J Immunol.* (2008) 181:1767–79. doi: 10.4049/jimmunol.181.3.1767
57. Zeng F, Dai C, Cai P, Wang J, Xu L, Li J, et al. A comparison study of SARS-CoV-2 IgG antibody between male and female COVID-19 patients: a possible reason underlying different outcome between sex. *J Med Virol.* (2020). (in press). doi: 10.1002/jmv.25989
58. Schurz H, Salie M, Tromp G, Hoal EG, Kinnear CJ, Möller M. The X chromosome and sex-specific effects in infectious disease susceptibility. *Human Genomics.* (2019) 13:2. doi: 10.1186/s40246-018-0185-z
59. Chamekh M, Casimir G. Editorial: sexual dimorphism of the immune inflammatory response in infectious and non-infectious diseases. *Front Immunol.* (2019) 10:107. doi: 10.3389/fimmu.2019.00107
60. Spolarics Z. The X-files of inflammation: cellular mosaicism of X-linked polymorphic genes and the female advantage in the host response to injury and infection. *Shock.* (2007) 27:597–604. doi: 10.1097/SHK.0b013e31802e40bd
61. Wang J, Syrett CM, Kramer MC, Basu A, Atchison ML, Anguera MC. Unusual maintenance of X chromosome inactivation predisposes female lymphocytes for increased expression from the inactive X. *Proc Natl Acad Sci USA.* (2016) 113:E2029–38. doi: 10.1073/pnas.1520113113
62. Oghumu S, Varikuti S, Stock JC, Volpedo G, Saljoughian N, Terrazas CA, et al. Cutting Edge: CXCR3 escapes X chromosome inactivation in t cells during infection: potential implications for sex differences in immune responses. *J Immunol.* (2019) 203:789–94. doi: 10.4049/jimmunol.1800931
63. Fan H, Dong G, Zhao G, Liu F, Yao G, Zhu Y, et al. Gender differences of B cell signature in healthy subjects underlie disparities in incidence and course of SLE related to estrogen. *J Immunol Res.* (2014) 2014:814598. doi: 10.1155/2014/814598
64. Wildbaum G, Netzer N, Karin N. Plasmid DNA encoding IFN-gamma-inducible protein 10 redirects antigen-specific T cell polarization and suppresses experimental autoimmune encephalomyelitis. *J Immunol.* (2002) 168:5885–92. doi: 10.4049/jimmunol.168.11.5885
65. Sarmiento L, Svensson J, Barchetta I, Giwercman A, Cilio CM. Copy number of the X-linked genes TLR7 and CD40L influences innate and adaptive immune responses. *Scand J Immunol.* (2019) 90:e12776.
66. Souyris M, Cenac C, Azar P, Daviaud D, Canivet A, Grunenwald S, et al. TLR7 escapes X chromosome inactivation in immune cells. *Sci Immunol.* (2018) 3:8855. doi: 10.1126/sciimmunol.aap8855
67. Laffont S, Rouquié N, Azar P, Seillet C, Plumas J, Aspord C, et al. Chromosome complement and estrogen receptor signaling independently contribute to the enhanced TLR7-mediated IFN-α production of plasmacytoid dendritic cells from women. *J Immunol.* (2014) 193:5444–52. doi: 10.4049/jimmunol.1303400
68. Harris VM, Harley ITW, Kurien BT, Koelsch KA, Scofield RH. Lysosomal pH is regulated in a sex dependent manner in immune cells expressing CXorf21. *Front Immunol.* (2019) 10:578. doi: 10.3389/fimmu.2019.00578
69. Odhams CA, Roberts AL, Vester SK, Duarte CST, Beales CT, Clarke AJ, et al. Interferon inducible X-linked gene CXorf21 may contribute to sexual dimorphism in systemic lupus erythematosus. *Nat Commun.* (2019) 10:1–15. doi: 10.1038/s41467-019-10106-2
70. Elgueta R, Benson MJ, de Vries VC, Wasiuk A, Guo Y, Noelle RJ. Molecular mechanism and function of CD40/CD40L engagement in the immune system. *Immunol Rev.* (2009) 229:782. doi: 10.1111/j.1600-065X.2009.00782.x
71. Lefrançois L, Olson S, Masopust D. A critical role for Cd40–Cd40 ligand interactions in amplification of the mucosal Cd8 T cell response. *J Exp Med.* (1999) 190:1275–84.
72. Ip WKE, Chan KH, Law HKW, Tso GHW, Kong EKP, Wong WHS, et al. Mannose-binding lectin in severe acute respiratory syndrome coronavirus infection. *J Infect Dis.* (2005) 191:1697–704. doi: 10.1086/429631
73. Schneider-Hohendorf T, Görlich D, Savola P, Kelkka T, Mustjoki S, Gross CC, et al. Sex bias in MHC I-associated shaping of the adaptive immune system. *Proc Natl Acad Sci USA.* (2018) 115:2168–73. doi: 10.1073/pnas.1716146115
74. Verma UN, Yamamoto Y, Prajapati S, Gaynor RB. Nuclear role of I kappa B Kinase-gamma/NF-kappa B essential modulator (IKK gamma/NEMO) in NF-kappa B-dependent gene expression. *J Biol Chem.* (2004) 279:3509–15. doi: 10.1074/jbc.M309300200
75. Liao Q-J, Ye L-B, Timani KA, Zeng Y-C, She Y-L, Ye L, et al. Activation of NF-kappaB by the full-length nucleocapsid protein of the SARS coronavirus. *Acta Biochim Biophys Sin (Shanghai).* (2005) 37:607–12. doi: 10.1111/j.1745-7270.2005.00082.x
76. Sun BK, Tsao H. X-Chromosome inactivation and skin disease. *J Investigat Dermatol.* (2008) 128:2753–9. doi: 10.1038/jid.2008.145
77. Martinez-Pomar N, Munoz-Saa I, Heine-Suner D, Martin A, Smahi A, Matamoros N. A new mutation in exon 7 of NEMO gene: late skewed X-chromosome inactivation in an incontinentia pigmenti female patient with immunodeficiency. *Hum Genet.* (2005) 118:458. doi: 10.1007/s00439-005-0068-y
78. Kenwrick S, Woffendin H, Jakins T, Shuttleworth SG, Mayer E, Greenhalgh L, et al. Survival of male patients with incontinentia pigmenti carrying a lethal mutation can be explained by somatic mosaicism or Klinefelter syndrome. *Am J Hum Genet.* (2001) 69:1210–7. doi: 10.1086/324591
79. Tommasini A, Ferrari S, Moratto D, Badolato R, Boniotto M, Pirulli D, et al. X-chromosome inactivation analysis in a female carrier of FOXP3 mutation. *Clin Exp Immunol.* (2002) 130:127–30.
80. Mercer F, Unutmaz D. The biology of FoxP3: a key player in immune suppression during infections, autoimmune diseases and cancer. *Adv Exp Med Biol.* (2009) 665:47–59.
81. Lu L, Barbi J, Pan F. The regulation of immune tolerance by FOXP3. *Nat Rev Immunol.* (2017) 17:703–17. doi: 10.1038/nri.2017.75
82. Cecere TE, Todd SM, LeRoith T. Regulatory T cells in arterivirus and coronavirus infections: do they protect against disease or enhance it? *Viruses.* (2012) 4:833–46. doi: 10.3390/v4050833
83. Dandekar AA, Perlman S. Immunopathogenesis of coronavirus infections: implications for SARS. *Nat Rev Immunol.* (2005) 5:917–27. doi: 10.1038/nri1732
84. Cui C, Yang W, Shi J, Zhou Y, Yang J, Cui Q, et al. Identification and analysis of human sex-biased MicroRNAs. *Genom Proteom Bioinform.* (2018) 16:200–11. doi: 10.1016/j.gpb.2018.03.004
85. Lu M, Zhang Q, Deng M, Miao J, Guo Y, Gao W, et al. An analysis of human MicroRNA and disease associations. *PLoS One.* (2008) 3:e3420. doi: 10.1371/journal.pone.0003420
86. Swaminathan G, Martin-Garcia J, Navas-Martin S. RNA viruses and microRNAs: challenging discoveries for the 21st century. *Physiol Genomics.* (2013) 45:1035–48. doi: 10.1152/physiolgenomics.00112.2013
87. Tahamtan A, Inchley CS, Marzban M, Tavakoli-Yaraki M, Teymoori-Rad M, Nakstad B, et al. The role of microRNAs in respiratory viral infection: friend or foe? *Rev Med Virol.* (2016) 26:389–407. doi: 10.1002/rmv.1894

88. Markle JG, Fish EN. SeXX matters in immunity. *Trends Immunol.* (2014) 35:97–104. doi: 10.1016/j.it.2013.10.006
89. Klein SL, Flanagan KL. Sex differences in immune responses. *Nat Rev Immunol.* (2016) 16:626–38. doi: 10.1038/nri.2016.90
90. Lotter H, Altfeld M. Sex differences in immunity. *Semin Immunopathol.* (2019) 41:133–5. doi: 10.1007/s00281-018-00728-x
91. Zazara DE, Arck PC. Developmental origin and sex-specific risk for infections and immune diseases later in life. *Semin Immunopathol.* (2019) 41:137–51. doi: 10.1007/s00281-018-0713-x
92. Steeg Vom LG, Klein SL. SeXX Matters in Infectious Disease Pathogenesis. *PLoS Pathogens* (2016) 12:e1005374. doi: 10.1371/journal.ppat.1005374
93. vom Steeg LG, Klein SL. Sex and sex steroids impact influenza pathogenesis across the life course. *Semin Immunopathol.* (2019) 41:189–94. doi: 10.1007/s00281-018-0718-5
94. Moulton VR, Holcomb DR, Zajdel MC, Tsokos GC. Estrogen upregulates cyclic AMP response element modulator α expression and downregulates interleukin-2 production by human T lymphocytes. *Mol Med.* (2012) 18:370–8. doi: 10.2119/molmed.2011.00506
95. Rider V, Jones S, Evans M, Bassiri H, Afsar Z, Abdou NI. Estrogen increases CD40 ligand expression in T cells from women with systemic lupus erythematosus. *J Rheumatol.* (2001) 28:2644–9.
96. Bynoe MS, Grimaldi CM, Diamond B. Estrogen up-regulates Bcl-2 and blocks tolerance induction of naive B cells. *Proc Natl Acad Sci USA*. (2000) 97:2703–8. doi: 10.1073/pnas.040577497
97. Panchanathan R, Choubey D. Murine BAFF expression is up-regulated by estrogen and interferons: implications for sex bias in the development of autoimmunity. *Mol Immunol.* (2013) 53:15–23. doi: 10.1016/j.molimm.2012.06.013
98. Pozzilli P, Lenzi A. Testosterone, a key hormone in the context of COVID-19 pandemic. *Metab Clin Exp.* (2020) 108:154252. doi: 10.1016/j.metabol.2020.154252
99. Gubbels Bupp MR, Jorgensen TN. Androgen-induced immunosuppression. *Front Immunol.* (2018) 9:794. doi: 10.3389/fimmu.2018.00794
100. Tan IJ, Peeva E, Zandman-Goddard G. Hormonal modulation of the immune system—A spotlight on the role of progestogens. *Autoimmun Rev.* (2015) 14:536–42.
101. Wambier CG, Goren A, Vaño-Galván S, Ramos PM, Ossimetha A, Nau G, et al. Androgen sensitivity gateway to COVID−19 disease severity. *Drug Dev Res.* (2020). (in press). doi: 10.1002/ddr.21688
102. Glowacka I, Bertram S, Müller MA, Allen P, Soilleux E, Pfefferle S, et al. Evidence that TMPRSS2 activates the severe acute respiratory syndrome coronavirus spike protein for membrane fusion and reduces viral control by the humoral immune response. *J Virol.* (2011) 85:4122–34. doi: 10.1128/JVI.02232-10
103. Schirmer M, Smeekens SP, Vlamakis H, Jaeger M, Oosting M, Franzosa EA, et al. Linking the human gut microbiome to inflammatory cytokine production capacity. *Cell.* (2016) 167:1125–36.e8. doi: 10.1016/j.cell.2016.10.020
104. Domínguez-Díaz C, García-Orozco A, Riera-Leal A, Padilla-Arellano JR, Fafutis-Morris M. Microbiota and its role on viral evasion: is it with us or against us? *Front Cell Infect Microbiol.* (2019) 9:256. doi: 10.3389/fcimb.2019.00256
105. Pang IK, Iwasaki A. Control of antiviral immunity by pattern recognition and the microbiome. *Immunol Rev.* (2012) 245:209–26. doi: 10.1111/j.1600-065X.2011.01073.x
106. Kalantar-Zadeh K, Ward SA, Kalantar-Zadeh K, El-Omar EM. Considering the effects of microbiome and diet on SARS-CoV-2 infection: nanotechnology roles. *ACS Nano.* (2020) 14:5179–82. doi: 10.1021/acsnano.0c03402
107. Perlot T, Penninger JM. ACE2 - from the renin-angiotensin system to gut microbiota and malnutrition. *Microbes Infect.* (2013) 15:866–73. doi: 10.1016/j.micinf.2013.08.003
108. Flores R, Shi J, Fuhrman B, Xu X, Veenstra TD, Gail MH, et al. Fecal microbial determinants of fecal and systemic estrogens and estrogen metabolites: a cross-sectional study. *J Transl Med.* (2012) 10:253. doi: 10.1186/1479-5876-10-253
109. Vemuri R, Sylvia KE, Klein SL, Forster SC, Plebanski M, Eri R, et al. The microgenderome revealed: sex differences in bidirectional interactions between the microbiota, hormones, immunity and disease susceptibility. *Semin Immunopathol.* (2019) 41:265–75. doi: 10.1007/s00281-018-0716-7
110. Fransen F, van Beek AA, Borghuis T, Meijer B, Hugenholtz F, van der Gaast-de Jongh C, et al. The impact of gut microbiota on gender-specific differences in immunity. *Front Immunol.* (2017) 8:754. doi: 10.3389/fimmu.2017.00754
111. Lotter H, Helk E, Bernin H, Jacobs T, Prehn C, Adamski J, et al. Testosterone increases susceptibility to amebic liver abscess in mice and mediates inhibition of IFNγ secretion in natural killer T cells. *PLoS One.* (2013) 8:e55694. doi: 10.1371/journal.pone.0055694
112. Flak MB, Neves JF, Blumberg RS. Welcome to the microgenderome. *Science.* (2013) 339:1044–5. doi: 10.1126/science.1236226
113. Yurkovetskiy L, Burrows M, Khan AA, Graham L, Volchkov P, Becker L, et al. Gender bias in autoimmunity is influenced by microbiota. *Immunity.* (2013) 39:400–12. doi: 10.1016/j.immuni.2013.08.013
114. Walls AC, Park Y-J, Tortorici MA, Wall A, McGuire AT, Veesler D. Structure, function, and antigenicity of the SARS-CoV-2 spike glycoprotein. *Cell.* (2020) 181:281–92.e6. doi: 10.1016/j.cell.2020.02.058
115. Yan R, Zhang Y, Li Y, Xia L, Guo Y, Zhou Q. Structural basis for the recognition of SARS-CoV-2 by full-length human ACE2. *Science.* (2020) 367:1444–8. doi: 10.1126/science.abb2762
116. Verdecchia P, Cavallini C, Spanevello A, Angeli F. The pivotal link between ACE2 deficiency and SARS-CoV-2 infection. *Eur J Intern Med.* (2020) 76:14–20. doi: 10.1016/j.ejim.2020.04.037
117. Imai Y, Kuba K, Rao S, Huan Y, Guo F, Guan B, et al. Angiotensin-converting enzyme 2 protects from severe acute lung failure. *Nature.* (2005) 436:112–6. doi: 10.1038/nature03712
118. Liu J, Ji H, Zheng W, Wu X, Zhu JJ, Arnold AP, et al. Sex differences in renal angiotensin converting enzyme 2 (ACE2) activity are 17β-oestradiol-dependent and sex chromosome-independent. *Biol Sex Differ.* (2010) 1:6. doi: 10.1186/2042-6410-1-6
119. Yan T, Xiao R, Lin G. Angiotensin-converting enzyme 2 in severe acute respiratory syndrome coronavirus and SARS-CoV-2: a double-edged sword? *FASEB J.* (2020) 34:6017–26. doi: 10.1096/fj.202000782
120. Sama IE, Ravera A, Santema BT, van Goor H, Ter Maaten JM, Cleland JGF, et al. Circulating plasma concentrations of angiotensin-converting enzyme 2 in men and women with heart failure and effects of renin–angiotensin–aldosterone inhibitors. *Eur Heart J.* (2020) 41:1810–7. doi: 10.1093/eurheartj/ehaa373
121. Li M-Y, Li L, Zhang Y, Wang X-S. Expression of the SARS-CoV-2 cell receptor gene ACE2 in a wide variety of human tissues. *Infect Dis Poverty.* (2020) 9:45. doi: 10.1186/s40249-020-00662-x
122. Pinto BG, Oliveira AE, Singh Y, Jimenez L, Goncalves AN, Ogava RL, et al. ACE2 expression is increased in the lungs of patients with comorbidities associated with severe COVID-19. *medRxiv.* (2020). . doi: 10.1101/2020.03.21.20040261
123. La Vignera S, Cannarella R, Condorelli RA, Torre F, Aversa A, Calogero AE. Sex-specific SARS-CoV-2 mortality: among hormone-modulated ACE2 expression, risk of venous thromboembolism and hypovitaminosis D. *Int J Mol Sci.* (2020) 21:2948. doi: 10.3390/Ijms21082948
124. Douglas GC, O'Bryan MK, Hedger MP, Lee DKL, Yarski MA, Smith AI, et al. The novel angiotensin-converting enzyme (ACE) Homolog, ACE2, is selectively expressed by adult leydig cells of the testis. *Endocrinology.* (2004) 145:4703–11. doi: 10.1210/en.2004-0443
125. Dalpiaz PLM, Lamas AZ, Caliman IF, Ribeiro RF, Abreu GR, Moyses MR, et al. Sex hormones promote opposite effects on ACE and ACE2 activity, hypertrophy and cardiac contractility in spontaneously hypertensive rats. *PLoS One.* (2015) 10:e0127515. doi: 10.1371/journal.pone.0127515
126. Gupte M, Thatcher SE, Boustany-Kari CM, Shoemaker R, Yiannikouris F, Zhang X, et al. Angiotensin converting enzyme 2 contributes to Sex differences in the development of obesity hypertension in C57BL/6 Mice. *Arterioscl Thrombos Vascul Biol.* (2012) 32:1392–9. doi: 10.1161/ATVBAHA.112.248559
127. White MC, Fleeman R, Arnold AC. Sex differences in the metabolic effects of the renin-angiotensin system. *Biol Sex Differen.* (2019) 10:31.

128. Ma L, Xie W, Li D, Shi L, Mao Y, Xiong Y, et al. Effect of SARS-CoV-2 infection upon male gonadal function: a single center-based study. *medRxiv*. (2020). [Preprint]. doi: 10.1101/2020.03.21.20037267
129. Harper A, Flanagan KL. Effect of sex on vaccination outcomes: important but frequently overlooked. *Curr Opin Pharmacol.* (2018) 41:122–7. doi: 10.1016/j.coph.2018.05.009
130. Fischinger S, Boudreau CM, Butler AL, Streeck H, Alter G. Sex differences in vaccine-induced humoral immunity. *Semin Immunopathol.* (2019) 41:239–49. doi: 10.1007/s00281-018-0726-5

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 28 August 2020
doi: 10.3389/fimmu.2020.02075

The Metabolic Changes and Immune Profiles in Patients With COVID-19

Bing He[1†], Jun Wang[2,3†], Yudie Wang[2,3†], Juan Zhao[1†], Juan Huang[4], Yu Tian[2,3], Cheng Yang[2,3], Heng Zhang[1], Mingxia Zhang[1], Lixing Gu[2,4], Xiaocui Zhou[1] and Jingjiao Zhou[2,3*]*

[1] Department of Pediatrics, Renmin Hospital of Wuhan University, Wuhan, China, [2] Department of Biology and Genetics, College of Life Sciences and Health, Wuhan University of Science and Technology, Wuhan, China, [3] Institute of Biology and Medicine, The College of Life Sciences and Health, Wuhan University of Science and Technology, Wuhan, China, [4] Department of Medicine, Hubei Maternal and Child Health Hospital, Wuhan, China

To explore the metabolic changes and immune profiles in patients with COVID-19, we analyzed the data of patients with mild and severe COVID-19 as well as young children with COVID-19. Of the leukocytes, 47% (IQR, 33–59) were lymphocytes [2.5 $\times$ 10^9/L (IQR, 2.2–3.3)], and monocytes were 0.51 $\times$ 10^9/L (IQR, 0.45–0.57) in young children with COVID-19. In 32 mild COVID-19 patients, circulating monocytes were 0.45 $\times$ 10^9/L (IQR, 0.36–0.64). Twenty-one severe patients had low PO_2 [57 mmHg (IQR, 50–73)] and SO_2 [90% (IQR, 86–93)] and high lactate dehydrogenase [580 U/L (IQR, 447–696)], cardiac troponin I [0.07 ng/mL (IQR, 0.02–0.30)], and pro-BNP [498 pg/mL (IQR, 241–1,726)]. Serum D-dimer and FDP were 9.89 mg/L (IQR, 3.62–22.85) and 32.7 mg/L (IQR, 12.8–81.9), and a large number of RBC (46/μL (IQR, 4–242) was presented in urine, a cue of disseminated intravascular coagulation (DIC) in severe patients. Three patients had comorbidity with diabetes, and 18 patients without diabetes also presented high blood glucose [7.4 mmol/L (IQR, 5.9–10.1)]. Fifteen of 21 (71%) severe cases had urine glucose +, and nine of 21 (43%) had urine ketone body +. The increased glucose was partially caused by reduced glucose consumption of cells. Severe cases had extraordinarily low serum uric acid [176 μmol/L (IQR, 131–256)]. In the late stage of COVID-19, severe cases had extremely low $CD4^+$ T cells and $CD8^+$ T cells, but unusually high neutrophils [6.5 $\times$ 10^9/L (IQR, 4.8–9.6)], procalcitonin [0.27 ng/mL (IQR, 0.14–1.94)], C-reactive protein [66 mg/L (IQR, 25–114)] and an extremely high level of interleukin-6. Four of 21 (19%) severe cases had co-infection with fungi, and two of 21 (9%) severe cases had bacterial infection. Our findings suggest that, severe cases had acute respiratory distress syndrome (ARDS) I–III, and metabolic disorders of glucose, lipid, uric acid, etc., even multiple organ dysfunction (MODS) and DIC. Increased neutrophils and severe inflammatory responses were involved in ARDS, MODS, and DIC. With the dramatical decrease of T-lymphocytes, severe cases were susceptible to co-infect with bacteria and fungi in the late stage of COVID-19. In young children, extremely high lymphocytes and monocytes might be associated with the low morbidity of COVID-19. The significantly increased monocytes might play an important role in the recovery of patients with mild COVID-19.

Keywords: COVID-19, SARS-CoV-2, acute respiratory distress syndrome (ARDS), metabolic disorders, disseminated intravascular coagulation (DIC), multiple organ dysfunctions (MODS), Inflammatory responses

OPEN ACCESS

Edited by:
Ata Murat Kaynar, University of Pittsburgh, United States

Reviewed by:
Daria Trabattoni, University of Milan, Italy
Alka Khaitan, Indiana University Bloomington, United States

***Correspondence:**
Xiaocui Zhou
hgzq@163.com
Jingjiao Zhou
zhoujj@wust.edu.cn

[†]These authors have contributed equally to this work

Specialty section:
This article was submitted to Viral Immunology, a section of the journal Frontiers in Immunology

Received: *07 April 2020*
Accepted: *30 July 2020*
Published: *28 August 2020*

Citation:
He B, Wang J, Wang Y, Zhao J, Huang J, Tian Y, Yang C, Zhang H, Zhang M, Gu L, Zhou X and Zhou J (2020) The Metabolic Changes and Immune Profiles in Patients With COVID-19. Front. Immunol. 11:2075. doi: 10.3389/fimmu.2020.02075

INTRODUCTION

In December 2019, an unknown viral pneumonia emerged in Wuhan, China, and it then escalated into an unprecedented outbreak (1). Chinese authorities have identified a new type of coronavirus named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (2). On February 11, 2020, the infectious disease caused by this viral strain was officially named COVID-19 (coronavirus disease 2019) by the World Health Organization (WHO) (3). By March 12, COVID-19 had swept into at least 117 countries and killed more than 4,000 people, and WHO officially announced a pandemic of COVID-19 viral disease (4). As of June 21, 2020, COVID-19 cases have been confirmed in 214 countries and territories, and the total was up to 8,708,008, including 461,715 deaths (5).

So far, according to reported patients' data, some remarkable phenomena have been observed. First, only 1% of patients with COVID-19 were infants and young children, and very few young patients have developed severe COVID-19 (6). Leukocytes are the main immune cells to fight against pathogens, and the total leukocyte count is higher in young children than in adults (7). Moreover, the thymus gland of an infant is large and continues to grow throughout childhood. Thus, the thymus produces more than enough matures T-lymphocytes throughout the child's life (8). We explored whether the count and differential of leukocytes in infants and young children are associated with very low morbidity rates of COVID-19.

Second, from the epidemiology and clinical characteristics of COVID-19, 81% of patients were diagnosed as mild cases, and most mild cases can recover from COVID-19 infection (9). So, it could be that specific leukocytes contributed to the recovery of patients with mild COVID-19. Monocytes are important immune sentinel cells critical in the defense against viral infection in the blood. They achieve this via diverse mechanisms that include the detection of viruses, migration into infected tissues, differentiation into macrophages and dendritic cells, and pathogen clearance by phagocytosis and intracellular killing (10, 11). Besides monocytes, the effect of lymphocytes on mild COVID-19 cases is still unclear. In this study, 32 mild patients have been examined to explore the potential roles of monocytes and lymphocytes in the recovery of patients with mild COVID-19.

Third, according to an analysis of nearly 45,000 confirmed cases, 19% of patients with COVID-19 have been identified as severe cases and critically ill cases, involving severe pneumonia and metabolic disorders, developing into acute respiratory distress syndrome (ARDS), multiple organ dysfunctions (MODS), and even septic shock and death (9, 12). Some studies suggested that the immunopathogenesis after viral infection has been linked to the development of the disease into severe cases (13, 14). To explore the potential roles of immunopathogenesis in the progress of COVID-19 infection, 21 severe COVID-19 patients have been investigated to explore how the immunopathogenesis was involved in ARDS and metabolic disorders, even MODS, disseminated intravascular coagulation (DIC), and death.

In this study, we investigated mild cases and severe cases infected with SARS-CoV-2, as well as healthy young children and adults. Our multiple comparative analysis showed that not only is leukocyte composition different in healthy groups, these differences can also be found during various stages of SARS-CoV-2 infection. Our study suggests that monocytes, neutrophils, and T-lymphocytes are associated with the onset and progress of COVID-19 infection, and immunopathogenesis was involved in ARDS, metabolic disorders, and MODS in severe cases. This study increases our understanding of the immune responses during COVID-19 infection and provides support to develop novel, feasible, and effective treatments for COVID-19 infection.

MATERIALS AND METHODS

Research Sources: COVID-19 Patients and Healthy Individuals

COVID-19 infection was rapidly endemic in Wuhan, China, in January, 2020. Renmin Hospital of Wuhan University is at the very forefront of the fight against COVID-19. We collected the data of patients with COVID-19, including the clinical records, laboratory results and chest computed tomography (CT) scan images of mild and severe cases in the hospital. For comparison with COVID-19 cases, the data of 35 healthy adults and 31 young children have been collected from the Physical Examination Center of the Hospitals. These healthy individuals have no significant medical condition and were in stable physical condition at that time.

The data of patients with COVID-19 and healthy persons have been all reviewed by a group of professional doctors from the hospitals, including basic features, nucleic acid tests, clinical data, laboratory results, co-infection with other pathogens, CT images, and other primary data. The study design has been approved by the Ethics Committee of the hospital.

Diagnoses of SARS-CoV-2 Infection

Nasopharyngeal swab samples were collected from patients, and tested as soon as possible to increase the detection rate of SARS-CoV-2. Reverse transcription polymerase chain reaction (RT-PCR) kit (Daan Gene, Shenzhen, China) was used to detect the conserved genes of SARS-CoV-2, such as ORF1ab gene, N gene, and E gene with LightCycler 480 System (Roche, Switzerland). If two or more of these three targeted genes has been detected as positive or one gene has been detected positive in two different samples from the same patient, the result is considered as positive for SARS-CoV-2. Meanwhile, the results can also be analyzed in conjunction with the patient's chest CT images.

Laboratory Data Analysis of Complete Blood Cell Count, Coagulation Profile, and Metabolic Indicators

Blood samples were collected from patients for laboratory tests. Serum biochemical tests, including aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatine kinase (CK) and lactate dehydrogenase (LDH) were determined with

Cobas C501 Testing System (Roche, Germany). Procalcitonin (PCT) and cardiac troponin I (cTn I) were analyzed by CL-2000i Chemiluminescence Immunoassay System (Mindray, Shenzhen, China). Coagulation indicators were detecting with ACL TOP 700 Hemostasis Testing Systems (Werfen, USA). All the blood samples from healthy persons were used for comparison.

Blood Tests for Immune Cells and Inflammation Factors

To study the count and differential of lymphocytes, the blood samples from COVID-19 patients were stained with CD3, CD4, CD8, CD19, CD16, and CD56 antibodies (BD Multi-test IMK kit, USA) and were analyzed by BD FACSCanto II Flow Cytometer (BD, USA). Th1/Th2 kit (BD, USA) was used to quantitatively measure IL-2, IL-4, IL-10, TNF, and IFN-γ protein levels. To examine the effect of SARS-CoV-2 on the patients' humoral immune function, immunoglobulins (IgM, IgG, IgA, and IgE), complement 3 (C3) and complement 4 (C4) were tested (Siemens Healthineers, USA). C-reactive protein (CRP) and interleukin (IL-6) were measured for COVID-19 patients (Mindray, Shenzhen, China).

Detections for Co-infection With Other Pathogens

Serum samples of patients were collected and tested for the IgM of respiratory tract pathogens with Pneumoslide IgM kit (Vircell, Spain), including human respiratory syncytial virus, influenza A virus (subtypes H1N1 and H3N2), influenza B virus, parainfluenza virus 1/2/3, metapneumovirus, common coronavirus, Epstein-Barr virus, cytomegalovirus, rhinovirus, adenovirus, and bocavirus, as well as *Legionella pneumophila* serum type I, *Mycoplasma pneumonia*, and *Chlamydia pneumoniae*. Nasopharyngeal secretions were tested for nucleic acids of 13 respiratory pathogens (Health Gene Technologies, Ningbo, China). Sputum culture was performed to identify bacterial and fungal co-infection. The fungal examination was performed with Fungus (1-3)-β-D-Glucan kit (Dynamiker Biotechnology, Tianjin, China) and *Platelia aspergillus* Ag kit (Bio-rad, USA).

Statistical Analysis

Continuous measurements have been presented as median and interquartile range (IQR) and categorical variables as percentages. For assessing laboratory results, we also assessed whether the measurements were outside the normal range. Unpaired *t*-test with Welch's correction was used for comparison, and $p < 0.05$ and <0.01 were considered statistically significant and highly statistically significant, respectively. GraphPad Prism 8.0.2 (San Diego, CA, USA) and SPSS25.0 (IBM, Armonk, NY, USA) were used for all analyses.

RESULTS

The Clinical Characteristics and the Changes of Lymphocytes and Monocytes Presented in Patients With Mild COVID-19

Patients with fever and/or cough were admitted to hospital after February 1, 2020. Chest CT images indicated multiple patchy, ground-glass opacity in the lungs (**Figure 1A**). Thirty-two patients were further diagnosed as infected with SARS-CoV-2 by real-time RT-PCR. There were 17 men and 15 women, and the median age of these mild cases was 42. The clinical characteristics of mild patients were presented in **Supplementary Table S1**.

Compared with healthy adults, the count of leukocytes and neutrophils in mild COVID-19 patients did not increase, but the median percentage and count of lymphocytes were 26% (IQR, 19–34) and 1.2×10^9/L (IQR, 1.1–1.6), respectively, which were significantly less than those of healthy adults, 34% (IQR, 29–39) and 2.0×10^9/L (IQR, 1.8–2.5), respectively ($p < 0.001$). Interestingly, the median percentage and count of monocytes were 8.2% (IQR, 7.1–9.2) and 0.45×10^9/L (IQR, 0.36–0.64), which were significantly higher than those of healthy adults 6.3% (IQR, 5.5–7.1) and 0.39×10^9/L (IQR, 0.35–0.42) ($p \leq 0.001$) (**Table 1**). The significantly increased number of monocytes could play an important role in the recovery of patients with mild COVID-19.

The Exceptionally High Lymphocytes and Monocytes Might Be Associated With Low Morbidity of COVID-19 in Young Children

To investigate why infants and young children have low morbidity of COVID-19, we analyzed the clinical characteristics of young children with COVID-19, and collected the data of circulating leukocytes of young children with/without COVID-19. Comparative analyses showed that young children have much higher leukocyte counts [6.9×10^9/L (IQR, 6.1–8.1)] than adults. Of note, 51% (IQR, 42–58) of leukocytes are lymphocytes [3.4×10^9/L, (IQR, 2.5–4.6)] in young children. The median count of monocytes in young children is 0.46×10^9/L (IQR, 0.41–0.67), which is much higher than that of adults [0.39×10^9/L (IQR, 0.35–0.42)] ($p = 0.001$). Lymphocytes of young children with COVID-19 was a little lower than those of healthy children, but remained at a high level [2.5×10^9/L (IQR, 2.2–3.3)]. Young children with COVID-19 had a high level of monocytes [0.51×10^9/L (IQR, 0.45–0.57)] as well (**Table 2**). Such a high number of lymphocytes and monocytes has benefit to fight against SARS-CoV-2, which might be associated with the low morbidity of COVID-19 in young children.

Patients With Severe COVID-19 Suffered From Severe Acute Respiratory Syndrome (ARDS, I-III)

We collected and compared the data of 21 severe cases and 32 mild cases. Chest CT images of severe cases indicated that there was critically diffuse ground-glass opacity in both lungs.

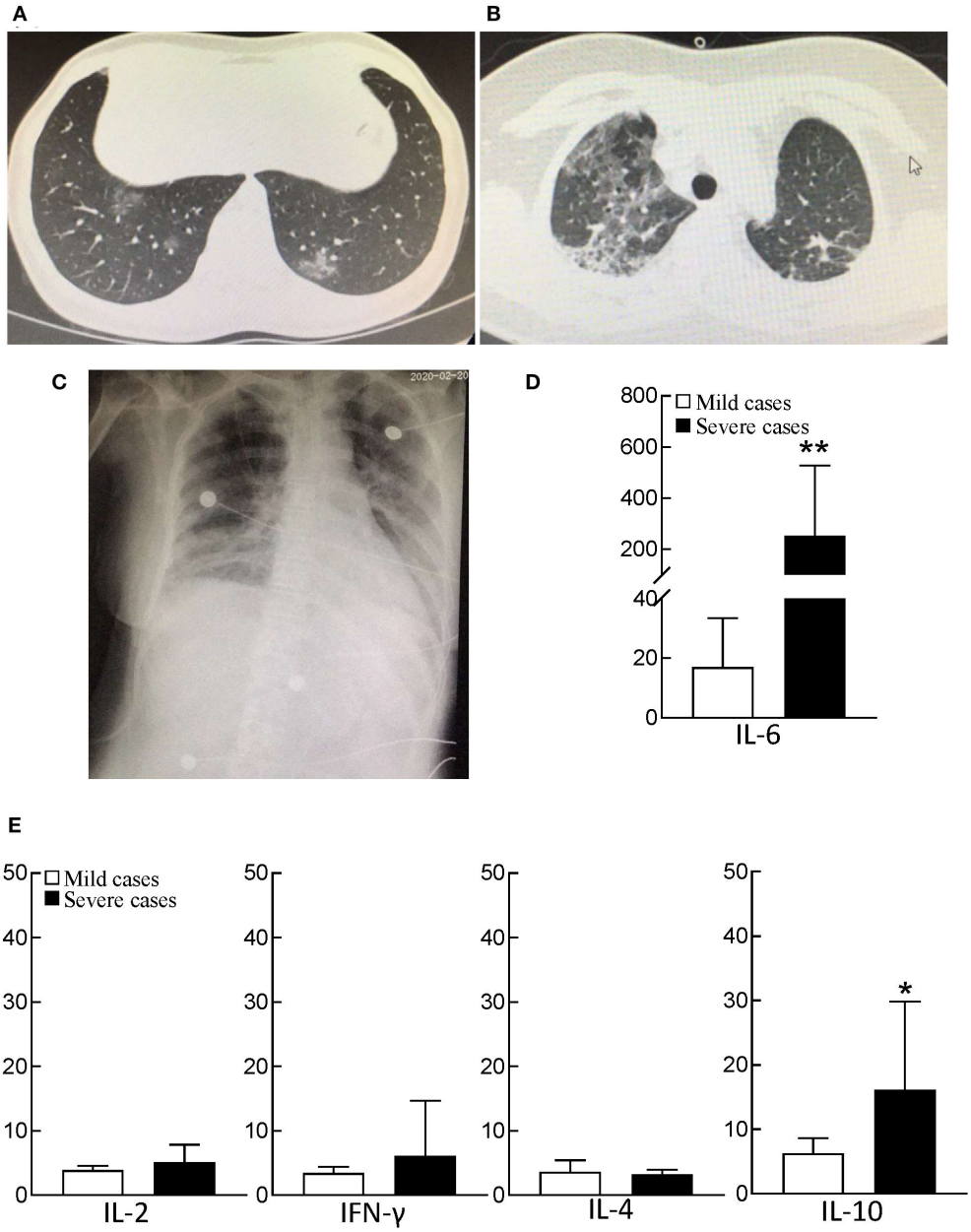


FIGURE 1 | CT and bedside chest X-ray images and serum cytokine concentrations of patients with COVID-19. **(A)** Chest CT image of mild patient showed small patchy, ground glass opacity in the lower lobes of both lungs. **(B)** Chest CT image of severe patient showed critically diffusing, ground glass opacity in the lungs, especially in right lung. **(C)** The critically ill patient's bedside chest X-ray showed the lung texture enhanced and the translucency decreased, and multiple patchy shadows in both lungs. **(D)** serum IL-6 concentration between mild patients ($n = 32$) and severe patients ($n = 21$). The normal range of IL-6 is ≤10 pg/ml. $^{**}p < 0.01$. **(E)** The analysis of Th1/Th2 cytokine panel between mild patients ($n = 32$) and severe patients ($n = 21$). The normal range of IL-2, IFN-γ, IL-4, and IL-10 are ≤11.4 pg/ml, 18 pg/ml, 12.9 pg/ml, and 5.9 pg/ml, respectively. $^{*}p < 0.05$.

A representative CT image is presented in **Figure 1B**. In bedside chest X-ray results of the critically ill patients, the translucency of both lungs was diffusely decreased, and a large area of patchy shadow appeared with uneven density. Tracheal intubation can be observed in the trachea and the heart shadow outline (**Figure 1C**). The clinical characteristics of severe patients were presented in **Supplementary Table S1**.

These CT and X-ray images showed that the primary and most significant changes were in the lower respiratory tract of patients with severe COVID-19. Among the respiratory indicators we measured, severe cases had lower partial pressure of oxygen (PO_2) and oxygen saturation (SO_2), 57 mmHg (IQR, 50–73) and 90% (IQR, 86–93), respectively, and suffered from different degrees of ARDS, I to III (**Table 3**).

TABLE 1 | Leukocyte count and differential of patients with COVID-19 and healthy adults.

	Normal range	Medium (IQR)			*P*-value[a]	*P*-value[b]	*P*-value[c]
		Healthy adults ($n = 35$)	Mild Covid-19 patients ($n = 32$)	Severe Covid-19 patients ($n = 21$)			
WBC, $\times 10^9$/L	3.5–9.5	6.2 (5.7–6.7)	4.7 (4.1–6.7)	7.6 (5.5–11.3)	0.3651	<0.001	<0.001
Lymphcyte, %	20–50	34 (29–39)	26 (19–34)	7 (4–10)	<0.001	<0.001	<0.001
Monocyte, %	3–10	6.3 (5.5–7.1)	8.2 (7.1–9.2)	4.5 (3.1–6.2)	<0.001	<0.001	<0.001
Neutrophil, %	40–75	56 (52–63)	64 (56–71)	88 (84–92)	0.004	<0.001	<0.001
Eosinophil, %	0.4–8.0	1.7 (1.0–2.4)	0.7 (0–2.6)	0 (0–0)	0.486	<0.001	<0.001
Basophil, %	0–1.0	0.7 (0.4–0.8)	0.3 (0.2–0.5)	0.1 (0–0.2)	0.001	<0.001	<0.001
Lymphcyte, $\times 10^9$/L	1.1–3.2	2.0 (1.8–2.5)	1.2 (1.1–1.6)	0.5 (0.3–0.8)	<0.001	<0.001	<0.001
Monocyte, $\times 10^9$/L	0.1–0.6	0.39 (0.35–0.42)	0.45 (0.36–0.64)	0.37 (0.21–0.51)	0.001	0.289	0.023
Neutrophil, $\times 10^9$/L	1.8–6.3	3.3 (3.1–4.3)	3.2 (2.3–4.6)	6.5 (4.8–9.6)	0.521	<0.001	<0.001
Eosinophil, $\times 10^9$/L	0.02–0.52	0.11 (0.05–0.15)	0.03 (0–0.12)	0 (0–0)	0.159	<0.001	<0.001
Basophil, $\times 10^9$/L	0–0.06	0.03 (0.03–0.05)	0.02 (0.01–0.03)	0.01 (0–0.02)	0.001	<0.001	0.019

COVID-19, coronavirus disease 2019; IQR, interquartile range; WBC, white blood cell.

[a] *P-values indicate differences between mild COVID-19 patients and healthy adults. $P < 0.05$ was considered statistically significant.*

[b] *P-values indicate differences between severe COVID-19 patients and healthy adults. $P < 0.05$ was considered statistically significant.*

[c] *P-values indicate differences between mild and severe COVID-19 patients. $P < 0.05$ was considered statistically significant.*

Severe COVID-19 Cases Had Metabolic Disorders, MODS, and Coagulation Disorders

Several cardiac parameters increased sharply, LDH [580 U/L (IQR, 447–696)], cardiac troponin I (cTnI) [0.07 ng/mL (IQR, 0.02–0.30)], as well as and pro-B-type natriuretic peptide (pro-BNP) [498 pg/mL (IQR, 241–1,726)], which indicated the heart function disorder, even heart failure in patients with severe COVID-19. Comparing the indicators of liver and kidney functions with those of mild cases, severe cases had higher AST [33 U/L (IQR, 26–64)] and glutamyltransferase (γ-GT) [45 U/L (IQR, 31–69)] and lower albumin (ALB) [32 g/L (IQR, 29–34)] and albumin/globulin ratio [1.1 (IQR, 0.9–1.3)] ($p < 0.01$); they also had higher urea [7.8 mmol/L (IQR, 5.9–9.1)] and lower Ca^{2+} (1.97 mmol/L (IQR, 1.89–2.05). Severe patients also had less of fibrinogen (FIB) [3.3 g/L (IQR 1.5–4.4)] and antithrombin III [78% (IQR, 71–85)]. For healthy people, the reference range of D-dimer is 0–0.55 mg/L, and the range for fibrin degradation product (FDP) is 0–5 mg/L. The severe cases had exceptionally high amounts of D-dimer and FDP, 9.89 mg/L (IQR, 3.62–22.85) and 32.7 mg/L (IQR, 12.8–81.9), respectively (**Table 3**). A high count of red blood cells (RBC) [46/μL (IQR, 4–242)] was presented in the urine of patients with severe COVID-19 (**Table 4**).

Increased glucose and low uric acid in blood should be noted here. The level of blood glucose was 5.2 mmol/L (IQR, 4.9–6.3) in 32 mild cases. Three of 21 (14%) severe cases had comorbidity with diabetes mellitus. Eighteen severe cases without comorbidity of diabetes also had high blood glucose [7.4 mmol/L (IQR, 5.9–10.1)]. Critically ill patients had extremely high levels of blood glucose [8.9 mmol/L (IQR, 6.8–12.9)]. Meanwhile, 15 of 21 (71%) severe cases had positive urine glucose +, and 9 of 21 (43%) severe cases had positive urine ketone body +. Additionally, serum uric acid was 275 μmol/L (IQR, 218–324) in mild cases, whereas an extraordinarily low level of serum uric acid [176 μmol/L (IQR, 131–256)] was found in severe cases (**Table 3**).

Severe Cases Had a Dramatical Decrease of T-lymphocytes and a Potentially High Risk of Co-infection

The total of leukocytes was 7.6×10^9/L (IQR, 5.5–11.3) in the peripheral blood of severe cases, which were much more than those in mild cases. Compared with mild cases, severe cases had low levels of monocytes [0.37×10^9/L (IQR, 0.21–0.51)]. However, the percentage and count of lymphocytes in severe cases were only 7% (IQR, 4–10) and 0.5×10^9/L (IQR, 0.3–0.8) respectively, which were significantly lower than those in mild cases (**Table 1**).

The subsets of lymphocytes were examined by flow cytometry, including natural killer (NK) cells ($CD16^+CD56^+$), B cells ($CD19^+$), and T cells ($CD3^+$). The results showed that severe cases had NK cells [63/μL (IQR, 26–109)] and B cells [91/μL (IQR, 54–181)], which was not a significant difference from the mild cases ($p > 0.05$). In addition, the functions of B cells and complements were tested, including IgM, IgG, IgA, IgE, C3, and C4, for both mild and severe COVID-19 cases. For severe cases, the values of IgM, C3, and C4 were slightly lower than those in mild cases, but these values were still within the normal range. However, compared with mild cases, severe cases had much lower levels of $CD4^+$ T cells and $CD8^+$ T cells, 146/μL [IQR, 107–277] and 59/μL (IQR, 33–109), respectively. The decrease of $CD8^+$ T cells was much more than that of $CD4^+$ T cells, and the ratio of $CD4^+$ T cells/$CD8^+$ T cells increased by 2.38 (IQR, 1.62–4.63) (**Table 4**). Further examination of Th1/Th2 cytokines also indicated that severe patients had normal levels of IL-2, and IFN-γ, as well as IL-4 in peripheral blood, but the level of IL-10 in severe patients was 4 times higher than normal (**Figure 1E**).

TABLE 2 | The clinical characteristics and leukocyte count and differential of young children with COVID-19.

	Median (IQR)		
	Healthy young children	**Healthy children with COVID-19**	***P*-value**
	(*n* = 31)	**(*n* = 16)**	
Age (Y)	4 (2–6)	6 (3–7)	
Gender (M/F)	16 (52%)/15 (48%)	10 (62%)/6 (38%)	
Mild case/Severe case	NA	1 (6%)/15 (94%)	
Signs and symptoms at admission			
Fever	NA	6 (38%)	
Cough	NA	12 (75%)	
Sputum	NA	0	
Shortness of breath	NA	1 (6%)	
Diarrhea	NA	0	
Treatment			
Antibiotic treatment	NA	10 (62%)	
Antiviral treatment	NA	10 (62%)	
hormone therapy	NA	1 (6%)	
Ventilation			
Non-invasive (face mask, etc)	NA	1 (6%)	
Mechanical ventilation	NA	0	
Discharged	NA	16 (100%)	
WBC, $\times 10^9$/L	6.9 (6.1–8.1)	5.6 (5.2–6.2)	0.007
Lymphcyte, %	51 (42–58)	47 (33–59)	0.239
Monocyte,%	6.7 (5.5–8.0)	8.7 (7.3–11.3)	0.027
Neutrophil, %	38 (33–46)	40 (26–65)	0.282
Eosinophil, %	2.1 (0.9–4.5)	2.6 (1.3–5.2)	0.646
Basophil,%	0.5 (0.2–0.7)	0.4 (0.3–0.5)	0.741
Lymphcyte, $\times 10^9$/L	3.4 (2.5–4.6)	2.5 (2.2–3.3)	0.008
Monocyte, $\times 10^9$/L	0.46 (0.41–0.67)	0.51 (0.45–0.57)	0.696
Neutrophil, $\times 10^9$/L	2.6 (2.1–3.0)	3.0 (2.0–4.1)	0.286
Eosinophil, $\times 10^9$/L	0.15 (0.06–0.32)	0.15 (0.04–0.29)	0.572
Basophil, $\times 10^9$/L	0.03 (0.02–0.05)	0.02 (0.01–0.04)	0.673

P-values indicate differences between young children with COVID-19 and healthy young children. P < 0.05 was considered statistically significant.

In this study, the clinical course of severe cases was over 3 weeks, and severe cases had a potentially high risk of co-infection with other pathogens due to critical exhaustion of $CD4^+$ and $CD8^+$ T cells. The respiratory tract pathogens were tested in severe cases, including 10 viruses, *Legionella pneumophila*, *Mycoplasma pneumoniae*, and *Chlamydia pneumoniae*, which were all negative. The fungal examinations, G assay and GM assay, were also performed in severe cases. The results of bacterial and fungal examinations indicated that four of 21 (19%) severe cases had co-infection with fungi, and two of 21 (9%) severe cases had co-infection with bacteria. A high number of white blood cells (WBC) [18/μL (IQR, 9–46)] was found in the urine of severe cases (**Table 4**).

Exceptionally High Neutrophils and Severe Inflammatory Responses Might Be Involved in ARDS, MODS and Coagulation Disorders

Further examinations showed that the median PCT was 0.27 ng/mL (IQR, 0.14–1.94) in severe cases, a cue of potential sepsis/septic shock. Among the inflammatory factors tested in severe cases, the median of CRP was 66 mg/L (IQR, 25–114), which was much higher than those in mild cases (**Table 4**). IL-6 slightly increased in mild cases, but exceptionally high level of IL-6 presented in severe cases, even 40 times higher than normal in some critically ill cases (**Figure 1D**). The release of the inflammatory factors triggered by SARS-CoV-2 replication and/or co-infection with bacteria and fungi, played important roles in the progress of COVID-19 infection.

In the late stage of the disease in severe COVID-19 cases, 88% (IQR, 84–92) of leukocytes were neutrophils [6.5×10^9/L (IQR, 4.8–9.6)] (**Table 1**). Previous studies showed that largely number of neutrophils triggered inflammatory responses and caused excessive organ injury in acute inflammatory disease, such as sepsis (15, 16). Exceptionally high neutrophil numbers might be involved in severe inflammatory responses and might be associated with ARDS, MODS, and even sepsis/septic shock, DIC, and death during the late stage of severe COVID-19 infection.

DISCUSSION

In this study, we first analyzed the clinical features and leukocyte differential of mild COVID-19 patients admitted to the hospital after February 1, 2020. Thirty-two mild cases, with a median age of 42 years, had recovered from COVID-19 infection. Our data showed that compared with healthy adults, patients with mild COVID-19 had lower lymphocytes in the acute stage, which was consistent with previous studies (12). However, mild COVID-19 cases had high counts of circulating monocytes [0.45×10^9/L (IQR, 0.36–0.64)]. In addition, mild patients had normal level of IL-4 and IL-10 in peripheral blood, but they had a 1–2-fold increase of IL-6. Monocytes/macrophages play very important roles in fighting against invading foreign viruses. Literature from the past 30 years has emphasized links among IL-6 and innate immune response, such as mononuclear phagocytes (10, 11, 17). For patients with mild COVID-19, a high monocyte count and slight increase of IL-6 might be helpful for eradicating the SARS-CoV-2 infection and were associated with recovery from COVID-19.

Based on the epidemiology and clinical characteristics of COVID-19, young children under six have the lowest morbidity rate, and very few young children with COVID-19 develop severe cases (6, 18). According to our comparative analysis, young children under six have highly circulating monocytes, and 51% (IQR, 42–58) of leukocytes are lymphocytes [3.4×10^9/L (IQR, 2.5–4.6)], including B-lymphocytes and T-lymphocytes. Lymphocytes of young children with COVID-19 was a little lower than those of healthy children, but remained at a high level [2.5×10^9/L (IQR, 2.2–3.3)]. Young children with COVID-19

TABLE 3 | The metabolic disorders and multi-organ dysfunctions in severe patients with COVID-19.

		Median (IQR)		
	Normal range	**Mild patients ($n = 32$)**	**Severe patients ($n = 21$)**	***P*-value[a]**
P02, mm Hg	80–100	85 (82–115)	57 (50–73)	0.003
S02, %	95–98	97 (95–98)	90 (86–93)	< 0.001
PC02, mm Hg	35–45	43 (39–47)	37 (33–40)	0.08
PH	7.35–7.45	7.39 (7.34–7.44)	7.46 (7.42–7.50)	0.04
BE, mmol/L	−3–3	2 (−1.3–4.3)	3.2 (−0.3–5)	0.28
cTnI, ng/ml	0–0.04	<0.01	0.07 (0.02–0.30)	
Mb, μg/L	0–110	29 (20–35)	54 (40–84)	< 0.001
CK, U/L	50–310	44 (31–82)	92 (50–153)	0.006
CK-MB, ng/ml	0–5	0.6 (0.5–0.8)	1.3 (0.9–2.5)	< 0.001
LOH, U/L	120–250	197 (170–229)	580 (447–696)	< 0.001
Pro-BNP, pg/ml	0–450	21 (8–97)	498 (241–1,726)	0.001
ALT, U/L	9–50	20 (11–33)	23 (17–44)	0.228
AST, U/L	15–35	18 (15–27)	33 (26–64)	0.007
ALP, U/L	45–125	57 (46–71)	73 (54–98)	0.001
y-GT, U/L	7–45	24 (14–42)	45 (31–69)	< 0.001
TP, g/L	65–85	65 (62–67)	61 (57–65)	0.006
ALB, g/L	40–55	42 (37–44)	32 (29–34)	< 0.001
A/G	1.2–2.4	1.7 (1.5–2.1)	1.1 (0.9–1.3)	< 0.001
TBIL, μmol/L	0–23	8.3 (6.5–11.2)	13 (8.5–17.6)	0.027
DBIL, μmol/L	0–8	2.8 (2.2–4.2)	5.1 (3.5–7.9)	< 0.001
Glucose, mmol/L	3.9–6.1	5.2 (4.9–6.3)	7.4 (5.9–10.1)	< 0.001
Uric acid, μmol/L	208–428	275 (218–324)	176 (131–256)	< 0.001
Cr, μmol/L	57–97	57 (49–69)	56 (50–66)	0.377
Urea, mmol/L	3.1–8	3.8 (3.3–4.3)	7.8 (5.9–9.1)	< 0.001
GFR, mL/min	>90	119 (112–122)	101 (93–109)	< 0.001
Na+, mmol/L	135–145	141 (139–143)	141 (138–145)	0.598
K+, mmol/L	3.5–5.5	3.9 (3.6–4.2)	3.7 (3.4–4.1)	0.461
Cl-, mmol/L	99–110	105 (103–107)	105 (101–107)	0.818
Ca2+, mmol/L	2.11–2.52	2.19 (2.11–2.25)	1.97 (1.89–2.05)	< 0.001
Mg2+, mmol/L	0.75–1.02	0.84 (0.80–0.89)	0.86 (0.80–0.93)	0.642
D-dimer, mg/L	0–0.55	0.38 (0. 19–0.79)	9.89 (3.62–22.85)	< 0.001
FDP, mg/L	0–5	0.9 (0.3–2.8)	32.7 (12.8–81.9)	< 0.001
PT, s	9–13	12 (12–13)	13 (12–13)	0.132
PS, %	75–135	83 (74–87)	77 (68–87)	0.234
APTT, s	25–31.3	29.1 (26.6–30.5)	27.8 (25.9–33.6)	0.242
FIB, g/L	2–4	3.6 (2.9–5.0)	3.3 (1.5–4.4)	0.07
AT-III,%	80–120	92 (86–100)	78 (71–85)	< 0.001
Urine				
Urine glucose (+)	Negative	NA	15/21 (71%)	
Urine ketone body (+)	Negative	NA	9/21 (43%)	

PO_2, partial pressure of oxygen; SO_2 (%), oxygen saturation; PCO_2, partial pressure of carbon dioxide; BE, Base Excess; cTnI, high-sensitivity cardiac troponin I; Mb, myoglobin; CK, creatine kinase; CK-MB, creatine kinase isoenzyme; LDH, Lactate dehydrogenase; Pro-BNP, Pro-Brain-type natriuretic peptide; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; γ-GT, γ-glutamyl transpeptidase; TP, total protein; ALB, albumin; A/G, albumin-globulin ratio; TBIL, total bilirubin; DBIL, direct bilirubin; Cr, creatinine; GFR, glomerular filtration rate; Na^+, sodium; K^+, potassium; Cl^-, chloride; Ca^{2+}, calcium; Mg^{2+}, magnesium; FDP, fibrinogen degradation products; PT, prothrombin time; PS, prothrombin time activity; APTT, activated partial thromboplastin time; FIB, fibrinogen; AT-III, antithrombin III.

P-values indicate differences between mild patients and severe patients. $P < 0.05$ was considered statistically significant.

had a high level of monocytes [0.51 × 10^9/L (0.45–0.57)] as well. The intricate process of T-lymphocyte development in the thymus is essential in the formation and maintenance of the peripheral T-lymphocytes. The thymus of a young child is big, and has the function of maintaining the large amounts of T-lymphocytes in the peripheral blood (19, 20). Extremely high levels of circulating lymphocytes and monocytes would benefit to fight against SARS-CoV-2 infection, which

might be associated with the low morbidity of COVID-19 in young children.

To explore the metabolic changes and immune responses in the progress of COVID-19 cases, we investigated 21 patients with severe COVID-19 infection. The median age of these patients was 57, and the clinical course was more than 3 weeks. CT scan images showed multiple patchy ground-glass shadows in the left and right lungs. Bedside chest radiography of critically ill patients indicated that the brightness of both lungs was decreased and multiple patchy shadows were observed. These clinical characteristics of severe cases are very similar to those reported in previous studies (21, 22). The 21 severe COVID-19 cases had ARDS I to III, and had extremely high levels of cTnI, LDH, and pro-BNP, a marker of severe cardiac dysfunction and even heart failure. Besides that, an extraordinarily low level of serum uric acid [176 μmol/L (131–256)] was found in severe cases. Uric acid is synthesized mainly in the liver and other tissues, which usually dissolves in the blood, and is removed from the body through urine. The extraordinarily low level of serum uric acid might indicate that potential liver and/or rental metabolism dysregulated in severe patients.

Among 21 severe cases, three patients had the comorbidity of diabetes, and other patients also had very high blood glucose [7.4 mmol/L (IQR, 5.9–10.1)]. Meanwhile, 15 out of 21 (71%) severe patients has positive of urine glucose (+), and nine out of 21 (43%) severe patients had positive of urine ketone body (+). The increased glucose of blood and urine was partially caused by the reduced glucose consumption of cells in severe patients. We need to pay attention to the high risks of metabolic syndromes mediated by high blood glucose, high urine glucose and urine ketone bodies. Dramatically high level of D-dimers [9.89 mg/L (IQR, 3.62–22.85)] and FDP [32.7 mg/L (IQR, 12.8–81.9)] were found in severe patients. A large amount of RBC [46/μL (IQR, 4–242)] was in urine of severe patients. These results showed that severe coagulation disorders, even DIC, occurred in these severe cases.

We further investigated immune responses in patients with severe COVID-19. First, different subpopulations of lymphocytes were investigated. The percentage and count of B cells and NK cells did not obviously change, which is consistent with the results from a previous report (23). The results of IgM/IgG/IgA/IgE, C3 and C4 also indicated that B cells and complements held normal functions. However, compared with mild cases, severe COVID-19 cases had lower levels of $CD4^+$ T cells [146/μL (IQR, 107–277)] and an even more significant reduction in $CD8^+$ T cells [only 59/μL (IQR, 33–109)], which has a sharper drop than $CD4^+$ T cell. We further analyzed Th1/Th2 panel, in severe patients, Th1 cytokines (IL-2 and IFN-γ) were in the normal range, but IL-10, one of Th2 cytokines, was about four times higher than normal. Previous studies presented that in severe patients, $CD4^+$ T cells and $CD8^+$ T cells highly expressed the exhaustion markers, including NKG2A, PD-1, and Tim-3 (24, 25). The dramatical decrease and functional exhaustion of $CD4^+$ T cells and $CD8^+$ T cells represents an important immunological characteristic of severe COVID-19 infection. Following the exhaustion of T cells, severe cases had high potential for co-infection with other pathogens. In this study, 4 of 21 (19%) severe patients had co-infection with fungi, and two of 21 (9%) severe patients had bacterial co-infection.

TABLE 4 | Immune and inflammatory profiles of patients with COVID-19.

		Median (IQR)		
	Normal range	**Mild patients ($n = 32$)**	**Severe patients ($n = 21$)**	***P*-value**
Lymphocytes				
CD3+, %	56–86	69 (66–77)	56 (49–66)	< 0.001
CD3+, /μL	723–2,737	794 (586–1,112)	221 (168−414)	< 0.001
CD4+, %	33–58	40 (36–46)	38 (27–46)	0.043
CD4+, /μL	404–1,612	433 (318−651)	146 (107–277)	< 0.001
CD8+, %	13–39	26 (22–32)	15 (9–24)	< 0.001
CD8+, /μL	220–1,129	297 (230–388)	59 (33–109)	< 0.001
CD4+/CD8+	0.9–2.0	1.45 (1.24–1.80)	2.38 (1.62–4.63)	< 0.001
CD19+, %	5–22	13 (9–19)	23 (13–33)	< 0.001
CD19+, /μL	80–616	125 (88−237)	91 (54–181)	0.123
CD16+CD56+, %	5–26	12 (9–18)	16 (10–19)	0.098
CD016+ CD56+, /μL	84–724	128 (87–213)	63 (26–109)	0.061
Humoral immunity				
Serum gobulin, g/L	20–40	22 (21–26)	30 (26–33)	< 0.001
IgM, g/L	0.4–2.3	1.0 (0.8−1.2)	0.7 (0.6–0.8)	0.027
IgG, g/L	7.0–16.0	11.2 (10.2–16.0)	16.6 (13.7–21.4)	0.016
IgA, g/L	0.7–4.0	2.4 (1.9–3.0)	2.3 (1.5–2.7)	0.263
IgE, IU/ml	<100	92 (55–170)	112 (75–191)	0.339
C3, g/L	0.9–1.8	1.1 (0.9–1.2)	0.9 (0.7–1.0)	0.002
C4, g/L	0.1–0.4	0.3 (0.2–0.3)	0.2 (0.1–0.2)	0.062
Inflammatory responses				
CRP, mg/L	<10	24 (11–51)	66 (25–114)	0.003
PCT, ng/ml	<0.1	0.05 (0.03–0.07)	0.27 (0.14–1.94)	0.02
Urine				
RBC, /μL	0–10	NA	46 (4–242)	
WBC, /μL	0–12	NA	18 (9–46)	

IgM, Immunoglobulin M; IgG, Immunoglobulin G; IgA, Immunoglobulin A; IgE, Immunoglobulin E; C3, complement C3; C4, complement C4; CRP, C-reactive protein; PCT, procalcitonin.

P-values indicate differences between mild and severe patients. P < 0.05 was considered statistically significant.

Twenty-one severe cases had a high level of PCT and CRP, 0.27 ng/mL (IQR, 0.14–1.94) and 66 mg/L (IQR, 25–114), respectively. IL-6 was much higher than normal in severe cases, even 40 times higher than normal in some critically ill cases. With SARS-CoV-2 replication and/or co-infection with bacteria and fungi, severe inflammatory responses played important roles in the progress of severe COVID-19 infection. In the late stage of severe COVID-19, 88% (IQR, 84–92) of leukocytes were neutrophils [6.5×10^9/L (IQR, 4.8–9.6). A high number of WBC [18/μL (IQR, 9–46)] was presented in urine of severe patients. Previous studies suggest that, in sepsis, a large number of neutrophil and the formation of neutrophil extracellular traps (NET) triggered severe inflammatory responses and excessive tissue damage (15, 16, 26). The significant increase in neutrophils might be

involved in severe inflammatory responses and MODS, even DIC and death in severe COVID-19 patients. Additionally, uric acid is the predominant anti-oxidant molecule in the plasma and respiratory tract, and is necessary for induction of type 2 immune responses. Uric acid plays a pivotal role in protecting against pathogen infections and autoimmune diseases (27, 28). Whether the decrease of serum uric acid is associated with the inflammatory responses in severe COVID-19 cases need to be explored.

There are several limitations to this study. First, we investigated 16 young children with COVID-19 and 53 adult cases, including 32 mild cases and 21 severe cases. More cases will need to be collected for comparative analysis of the difference between severe and critically ill patients. Second, more inflammatory cytokines and chemokines will be analyzed for severe and critically ill patients and will be further evaluated for inflammatory storm mediated ARDS, DIC, MODS, and coagulation disorders. Third, the mechanisms by which SARS-CoV-2 infection causes the reduction and functional exhaustion of $CD4^+$ T cells and $CD8^+$ T cells are still unclear. *In-vitro* and *in-vivo* experiments need to be performed to explore the mechanisms of T cell exhaustion.

In summary, our findings suggest that extremely high level of lymphocytes and monocytes could help hamper SARS-CoV-2 replication, which might be associated with the low morbidity of COVID-19 in infants and young children. A high number of monocytes would be helpful for removing SARS-CoV-2 and play an important role in the recovery of patients with mild COVID-19. In the late stage of the disease, severe cases suffered from ARDS, metabolic disorders, MODS and coagulation disorders. With dramatical decrease of $CD4^+$ T cells and $CD8^+$ T cells, extraordinarily increased neutrophils and severe inflammatory responses are involved in ARDS, MODS, and coagulation disorders and can even lead to DIC and death in severe cases. Whether the decrease of serum uric acid is associated with the inflammatory responses in severe COVID-19 cases needs to be further explored. These findings can not only greatly improve our understanding of metabolic and immunological characteristics, but also provide a mechanistic basis for the prevention and treatment of COVID-19 infection.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Renmin hospital of Wuhan University. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

JZho, XZ, and BH developed the concept and designed this study. JZho, XZ, BH, JW, LG, YW, JH, and JZha contributed the acquisition, analysis, and interpretation of data. JZho, BH, JW, YW, and LG contributed drafting of the manuscript and critical revision of the manuscript for important intellectual content. JW, YW, JZha, and CY conducted the statistical analysis. YT, HZ, JW, CY, JZha, JH, and MZ performed the administrative, technical, and material support duties. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the Special Project for the Biomedical Research and Development (118/1180011), and "Chutian Scholar" Program, Hubei Provincial Department of Education.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.02075/full#supplementary-material

REFERENCES

1. Li Q, Guan X, Wu P, Wang X, Zhou L, Tong Y, et al. Early transmission dynamics in Wuhan, China, of novel coronavirus-infected pneumonia. *N Engl J Med.* (2020) 382:1199–207. doi: 10.1056/NEJMoa2001316
2. Zhu N, Zhang D, Wang W, Li X, Yang B, Song J, et al. China Novel Coronavirus Investigating and Research Team. A novel coronavirus from patients with pneumonia in China, 2019. *N Engl J Med.* (2020) 382:727–733. doi: 10.1056/NEJMoa2001017
3. World Health Organization. *Novel Coronavirus (2019-nCoV) Situation Report-22.* Available online at: https://www.who.int/docs/default-source/coronaviruse/situation-reports/20200211-sitrep-22-ncov.pdf (accessed February 11, 2020)
4. World Health Organization. *Coronavirus disease 2019 (COVID-19) Situation Report-52.* Available online at: https://www.who.int/docs/default-source/coronaviruse/situation-reports/20200312-sitrep-52-covid-19.pdf (accessed March 12, 2020)
5. World Health Organization. *Coronavirus disease 2019 (COVID-19) Situation Report-153.* Available online at: https://www.who.int/docs/default-source/coronaviruse/situation-reports/20200621-covid-19-sitrep-153.pdf (accessed June 21, 2020)
6. Wu Z, McGoogan JM. Characteristics of and important lessons from the coronavirus disease 2019 (COVID-19) outbreak in China: summary of a report of 72314 cases from the Chinese Center for Disease Control and Prevention. *JAMA.* (2020) doi: 10.1001/jama.2020.2648
7. Rana SR, Castro OL, Haddy TB. Leukocyte counts in 7,739 healthy black persons: effects of age and sex. *Ann Clin Lab Sci.* (1985) 15:51–4.
8. Tulic MK, Andrews D, Crook ML, Charles A, Tourigny MR, Moqbel R, et al. Changes in thymic regulatory T-cell maturation from birth to puberty: differences in atopic children. *J Allergy Clin Immunol.* (2012) 129:199–206.e1-4. doi: 10.1016/j.jaci.2011.10.016
9. World Health Organization. *Coronavirus disease 2019 (COVID-19) situation report-41.* Available online at: https://www.who.int/docs/default-source/coronaviruse/situation-reports/20200301-sitrep-41-covid-19.pdf (accessed March 1, 2020)
10. de Jong E, Strunk T, Burgner D, Lavoie PM, Currie A. The phenotype and function of preterm infant monocytes: implications for susceptibility to infection. *J Leukoc Biol.* (2017) 102:645–56. doi: 10.1189/jlb.4RU0317-111R

11. Yewdell JW, Brooke CB. Monocytes, viruses and metaphors: hanging the Trojan horse. *Cell Cycle*. (2012) 11:1748–9. doi: 10.4161/cc.20311
12. Chen N, Zhou M, Dong X, Qu J, Gong F, Han Y, et al. Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study. *Lancet*. (2020) 395:507513. doi: 10.1016/S0140-6736(20)30211-7
13. Xu Z, Shi L, Wang Y, Zhang J, Huang L, Zhang C, et al. Pathological findings of COVID-19 associated with acute respiratory distress syndrome. *Lancet*. (2020) doi: 10.1016/S2213-2600(20)30076-X
14. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet*. (2020) 395:497–506. doi: 10.1016/S0140-6736(20)30183-5
15. Leliefeld PH, Wessels CM, Leenen LP, Koenderman L, Pillay J. The role of neutrophils in immune dysfunction during severe inflammation. *Crit Care*. (2016) 20:73. doi: 10.1186/s13054-016-1250-4
16. Zhang Q, Raoof M, Chen Y, Sumi Y, Sursal T, Junger W, et al. Circulating mitochondrial DAMPs cause inflammatory responses to injury. *Nature*. (2010) 464:104–7. doi: 10.1038/nature08780
17. Chomarat P, Banchereau J, Davoust J, Palucka K. IL-6 switches the differentiation of monocytes from dendritic cells to macrophages. *Nat Immunol*. (2000) 1:510–4. doi: 10.1038/ni.3153
18. Sun K, Chen J, Viboud C. Early epidemiological analysis of the coronavirus disease 2019 outbreak based on crowdsourced data: a population-level observational study. *Lancet*. (2020) 2: e201–8. doi: 10.1016/S2589-7500(20)30026-1
19. Park JE, Botting RA, Domínguez Conde C, Popescu DM, Lavaert M, Kunz DJ, et al. A cell atlas of human thymic development defines T cell repertoire formation. *Science*. (2020) 367:eaay3224. doi: 10.1126/science.aay3224
20. van der Weerd K, Dik WA, Schrijver B, Bogers AJ, Maat AP, van Nederveen FH, et al. Combined TCRG and TCRA TREC analysis reveals increased peripheral T-lymphocyte but constant intra-thymic proliferative history upon ageing. *Mol Immunol*. (2013) 53:302–12. doi: 10.1016/j.molimm.2012.08.019
21. Wang D, Hu B, Hu C, Zhu F, Liu X, Zhang J, et al. Clinical characteristics of 138 hospitalized patients with 2019 novel coronavirus-infected pneumonia in Wuhan, China. *JAMA*. (2020) 323:1061–9. doi: 10.1001/jama.2020.1585
22. Young BE, Ong SW, Kalimuddin S, Low JG, Tan SY, Loh J, et al. Epidemiologic features and clinical course of patients infected with SARS-CoV-2 in Singapore. *JAMA*. (2020) 323:1488–94. doi: 10.1001/jama.2020.3204
23. Liu J, Li S, Liu J, Liang B, Wang X, Wang H, et al. Longitudinal characteristics of lymphocyte responses and cytokine profiles in the peripheral blood of SARS-CoV-2 infected patients. *medRxiv*. (2020). doi: 10.1101/2020.02.16.20023671
24. Zheng M, Gao Y, Wang G, Song G, Liu S, Sun D, et al. Functional exhaustion of antiviral lymphocytes in COVID-19 patients. *Cell Mol Immunol*. (2020) 17:533–5. doi: 10.1038/s41423-020-0402-2
25. Chen Y, Diao B, Wang C, Chen X, Liu Y, Ning L, et al. Reduction and functional exhaustion of T cells in patients with coronavirus disease 2019 (COVID-19). *Front Immunol*. (2020) 11:827. doi: 10.3389/fimmu.2020.00827
26. Papayannopoulos V. Neutrophil extracellular traps in immunity and disease. *Nat Rev Immunol*. (2018) 18:134–47. doi: 10.1038/nri.2017.105
27. El Ridi R, Tallima H. Physiological functions and pathogenic potential of uric acid: a review. *J Adv Res*. (2017). doi: 10.1016/j.jare.2017.03.003
28. Hammad H, Lambrecht BN. Barrier epithelial cells and the control of type 2 immunity. *Immunity*. (2015) 43:29–40. doi: 10.1016/j.immuni.2015.07.007

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Public Health

SYSTEMATIC REVIEW
published: 02 September 2020
doi: 10.3389/fpubh.2020.554247

Research Collaboration and Outcome Measures of Interventional Clinical Trial Protocols for COVID-19 in China

Ya Gao[1†], Kelu Yang[2†], Ming Liu[1], Yamin Chen[2], Shuzhen Shi[1], Fengwen Yang[3] and Jinhui Tian[1,2,4*]*

[1] Evidence-Based Medicine Center, School of Basic Medical Sciences, Lanzhou University, Lanzhou, China, [2] Evidence-Based Nursing Center, School of Nursing, Lanzhou University, Lanzhou, China, [3] Evidence-Based Medicine Center, Tianjin University of Traditional Chinese Medicine, Tianjin, China, [4] Key Laboratory of Evidence-based Medicine and Knowledge Translation of Gansu Province, Lanzhou University, Lanzhou, China

OPEN ACCESS

Edited by:
Zisis Kozlakidis,
International Agency for Research on Cancer (IARC), France

Reviewed by:
Xian-Tao Zeng,
Wuhan University, China
Hong-Bing Xiang,
Huazhong University of Science and Technology, China

***Correspondence:**
Jinhui Tian
tianjh@lzu.edu.cn
Fengwen Yang
836029745@qq.com

[†]These authors have contributed equally to this work

Specialty section:
This article was submitted to Infectious Diseases, Surveillance, Prevention and Treatment, a section of the journal Frontiers in Public Health

Received: 21 April 2020
Accepted: 14 August 2020
Published: 02 September 2020

Citation:
Gao Y, Yang K, Liu M, Chen Y, Shi S, Yang F and Tian J (2020) Research Collaboration and Outcome Measures of Interventional Clinical Trial Protocols for COVID-19 in China. Front. Public Health 8:554247. doi: 10.3389/fpubh.2020.554247

Background: Research collaboration of registered clinical trials for Coronavirus Disease 2019 (COVID-19) remains unclear. This study aimed to analyze research collaboration and distribution of outcome measures in registered interventional clinical trials (ICTs) of COVID-19 conducted in China.

Methods: The International Clinical Trials Registry Platform, China Clinical Trials Registry, and Clinicaltrials.gov were searched to obtain COVID-19-registered ICTs up to May 25, 2020. Excel 2016 was used to perform a descriptive statistical analysis of the extracted information. VOSviewer 1.6.14 software was used to generate network maps for provinces and institutions and create density maps for outcomes.

Results: A total of 390 ICTs were included, and the number of daily registrations fluctuated greatly. From 29 provinces in China, 430 institutions contributed to the registration of ICTs. The top three productive provinces were Hubei (160/390, 41.03%), Shanghai (60/390, 15.38%), and Beijing (59/390, 15.13%). The top three productive institutions were Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology (30/390, 7.69%), Zhongnan Hospital of Wuhan University (18/390, 4.62%), and Wuhan Jinyintan Hospital (18/390, 4.62%). Collaborations between provinces and institutions were not close enough. There were many interventions, but many trials did not provide specific drugs and their dosage and treatment duration. The most frequently used primary outcome was Chest/lung CT (53/390, 13.59%), and the most frequently used secondary outcome was hospital stay (33/390, 8.46%). There was a large difference in the number of outcomes, the expression of some outcomes was not standardized, the measurement time and tools for some outcomes were not clear, and there was a lack of special outcomes for trials of traditional Chinese medicine.

Conclusions: Although there were some collaborations between provinces and institutions of the current COVID-19 ICT protocols in China, cooperation between regions should be further strengthened. The identified deficiencies in interventions and outcome measures should be given more attention by future researchers of COVID-19.

Keywords: COVID-19, SARS-CoV-2, clinical trials, protocol, research collaboration, outcome measures

INTRODUCTION

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a novel enveloped RNA betacoronavirus, has the characteristics of fast spread and strong infectivity (1–3). In late December 2019, the Coronavirus Disease 2019 (COVID-19) caused by SARS-CoV-2 first appeared, and it then quickly spread to various countries (4–6). On March 11, 2020, the World Health Organization (WHO) declared the outbreak of SARS-CoV-2 as a pandemic (7). As of July 12, 2020, a total of 12,552,765 confirmed cases were reported worldwide, including 561,617 deaths (8). To find an effective drug to treat COVID-19, medical workers and scientific researchers actively carry out research and have registered numerous clinical trials. Recently, scholars have assessed the characteristics and status quo of registered COVID-19 clinical trials (9, 10). However, no research has focused on the research collaboration of these registered clinical trials. This study was designed to evaluate the cooperation between institutions and the distribution of outcome measures in registered interventional clinical trials (ICTs) of COVID-19 conducted in China, to provide a reference for future researchers to register and carry out COVID-19 clinical trials.

MATERIALS AND METHODS

Data Sources

We systematically searched the International Clinical Trials Registry Platform (ICTRP, https://www.who.int/ictrp/en/), China Clinical Trials Registry (ChiCTR, http://www.chictr.org.cn), and ClinicalTrials.gov to obtain registered trials related to COVID-19. The searches were conducted initially on February 20, 2020 and updated on May 25, 2020. The search terms included severe acute respiratory syndrome coronavirus 2, SARS-CoV-2, new coronavirus, new coronary pneumonia, NCP, 2019-nCoV, COVID-19, novel corona virus, novel coronavirus, nCoV-2019, corona virus pneumonia disease 2019, novel coronavirus pneumonia, 2019 novel coronavirus, coronavirus disease 2019, and coronavirus disease-19.

Inclusion and Exclusion Criteria

We included registered ICTs of COVID-19 that conducted in China without restricting the types of interventions, comparisons, and outcomes. We excluded trials conducted outside China. Studies of basic science, diagnostic test, and epidemiological research as well as duplication and retracted records were also excluded.

Study Selection and Data Extraction

Two researchers (Y.G. and K.L.Y.) independently reviewed the records and screened out eligible ICTs according to the inclusion and exclusion criteria, and then proceeded to a cross-check. Conflicts were settled through discussions with a third reviewer (J.H.T.). We developed a data extraction form using Microsoft Excel 2016 (Microsoft Corp, Redmond, WA, www.microsoft.com) through discussions with the review team. Then, one author (Y.G., K.L.Y., or M.L.) extracted data from the included ICTs using the pre-defined form and a second reviewer (F.W.Y, or J.H.T.) checked the extracted data. The detailed data included: registration number, registration time, title, inclusion criteria, exclusion criteria, gender and age of the population, sample size, provinces, institutions, interventions, primary outcomes, and secondary outcomes.

Data Management and Analysis

For institutions, interventions, and outcomes with different expressions, we have processed them, leaving only a standardized name. Microsoft Excel 2016 (Microsoft Corp, Redmond, WA, www.microsoft.com) was used to perform descriptive statistical analysis of the extracted information. VOSviewer 1.6.14 (Leiden University, Leiden, Netherlands) software was utilized to extract provinces and institutions and generate corresponding cooperation network maps. Furthermore, we created density maps for high-frequency primary and secondary outcome measures. In this study, the nodes in the network map represented the analyzed elements (provinces and institutions), the size of the nodes reflected the frequency of elements, the colors of nodes and lines represented different clusters, and the links between nodes indicated the relationship of cooperation or co-occurrence (11–14). The parameters of the VOSviewer were as follows: counting method (fractional counting), ignore documents with many authors (maximum number of authors per document is 25).

RESULTS

Screening Results

A total of 3,541 records were retrieved through the systematic literature search, and 1,159 were non-interventional trials. After reading the detailed registration information, we further excluded 1,992 records for the following reasons: trials conducted outside China (n = 1,336), duplicate records (n = 609), retracted/terminated trials (n = 47). Finally, 390 ICTs were included for analysis. The flowchart of the screening process is provided in **Figure S1**.

General Characteristics of Included ICTs

The number of daily COVID-19 ICT registrations fluctuated considerably, and the maximum number of registrations per day was 13 (**Figure 1**). Six (1.54%) ICTs incorporated only males, and the remaining 384 (98.46%) ICTs included both males and females. A total of 74.87% of ICTs included adults (18 years and older), but 59 (15.13%) ICTs did not report the age of the included population. The total sample size of the 390 ICTs was 109,372, and the smallest sample size was only four; the maximum was 20,000, and the median was 100.

Provinces

A total of 29 provinces participated in the registration of COVID-19 ICTs. The number of ICTs conducted by one, two, three, four, five, and six provinces were 304/390 (77.95%), 61/390 (15.64%), 12/390 (3.08%), 4/390 (1.03%), 6/390 (1.54%), and 3/390 (0.77%), respectively. The top five productive provinces were Hubei

Abbreviations: COVID-19, Corona Virus Disease 2019; SARS-CoV-2, Severe acute respiratory syndrome coronavirus 2; ICTs, interventional clinical trials.

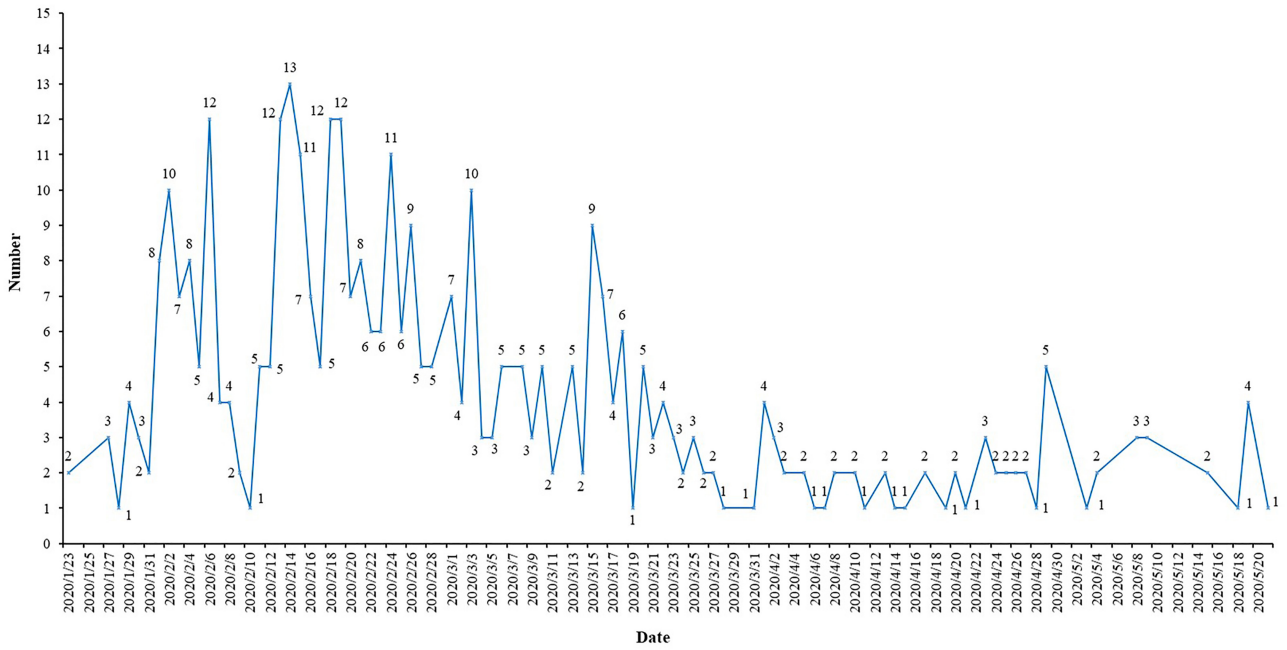


FIGURE 1 | Registration time for ICTs of COVID-19.

TABLE 1 | Provinces contributed to the registration of COVID-19 ICTs [N (%)].

Rank	Provinces	N (%)	Rank	Provinces	N (%)
1	Hubei	160 (41.03%)	16	Fujian	6 (1.54%)
2	Shanghai	60 (15.38%)	17	Liaoning	6 (1.54%)
3	Beijing	59 (15.13%)	18	Guizhou	5 (1.28%)
4	Guangdong	44 (11.28%)	19	Tianjin	4 (1.03%)
5	Zhejiang	34 (8.72%)	20	Hebei	3 (0.77%)
6	Sichuan	21 (5.38%)	21	Guangxi	2 (0.51%)
7	Jiangsu	18 (4.62%)	22	Inner Mongolia	2 (0.51%)
8	Henan	17 (4.36%)	23	Ningxia	2 (0.51%)
9	Anhui	13 (3.33%)	24	Shanxi	2 (0.51%)
10	Hunan	13 (3.33%)	25	Hainan	1 (0.26%)
11	Jiangxi	13 (3.33%)	26	Hong Kong	1 (0.26%)
12	Heilongjiang	11 (2.82%)	27	Jilin	1 (0.26%)
13	Shaanxi	11 (2.82%)	28	Xinjiang	1 (0.26%)
14	Shandong	8 (2.05%)	29	Yunnan	1 (0.26%)
15	Chongqing	7 (1.79%)			

(160/390, 41.03%), Shanghai (60/390, 15.38%), Beijing (59/390, 15.13%), Guangdong (44/390, 11.28%), and Zhejiang (34/390, 8.72%); the provinces participating in the registration of six to 21 ICTs were Sichuan (21/390, 5.38%), Jiangsu (18/390, 4.62%), Henan (17/390, 4.36%), Anhui (13/390, 3.33%), Hunan (13/390, 3.33%), Jiangxi (13/390, 3.33%), Heilongjiang (11/390, 2.82%), Shaanxi (11/390, 2.82%), Shandong (8/390, 2.05%), Chongqing (7/390, 1.79%), Fujian (6/390, 1.54%), and Liaoning (6/390, 1.54%). The remaining provinces participated in the registration of fewer than six ICTs, the detailed information is presented in **Table 1**.

A social network analysis of provinces revealed that 26 provinces formed a cooperative relationship. Hubei, located in the center of the network, had more collaborations with other provinces. Shanxi, Fujian, Hainan, and Guizhou were situated on the edge of the network and had little cooperation with other provinces. Xinjiang, Jilin, and Hong Kong did not cooperate with other provinces (**Figure 2**).

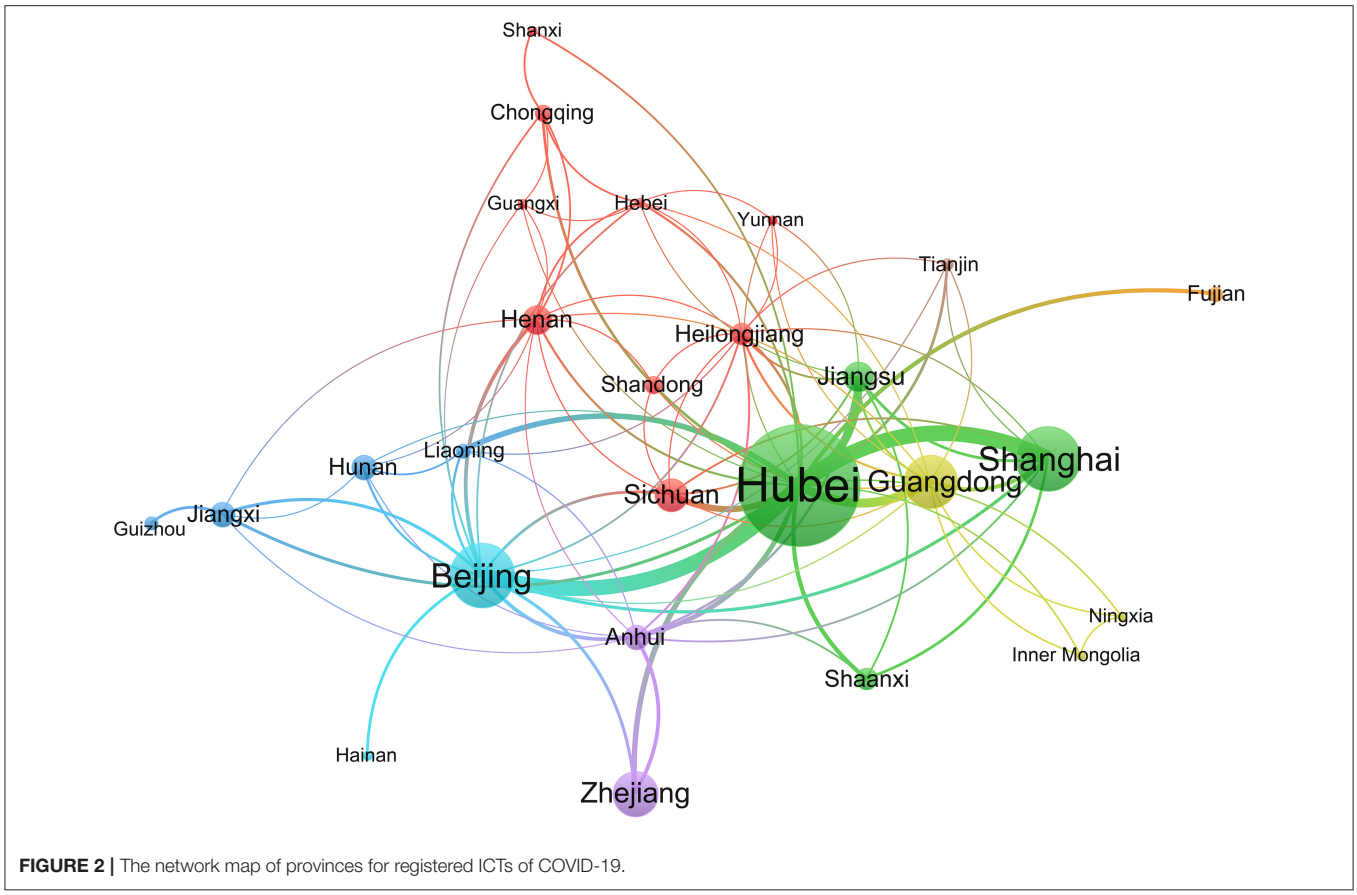


FIGURE 2 | The network map of provinces for registered ICTs of COVID-19.

Institutions

A total of 430 institutions contributed to the registration of COVID-19 ICTs. The number of ICTs conducted by one, two, three, four, five, six, seven, eight, nine, and more than nine institutions were 228/390 (58.46%), 78/390 (20.00%), 27/390 (6.92%), 15/390 (3.85%), 14/390 (3.59%), 10/390 (2.56%), 4/390 (1.03%), 4/390 (1.03%), 4/390 (1.03%), and 6/390 (1.54%), respectively. A total of 282/430 (65.58%) institutions participated in only one ICT, and 66/430 (15.35%) institutions participated in two ICTs. Institutions participating in the registration of more than 10 ICTs included Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology (30/390, 7.69%), Zhongnan Hospital of Wuhan University (18/390, 4.62%), Wuhan Jinyintan Hospital (18/390, 4.62%), Shanghai Public Health Clinical Center (17/390, 4.36%), Union Hospital, Tongji Medical College, Huazhong University of Science and Technology (14/390, 3.59%), the First Affiliated Hospital of Guangzhou Medical University (13/390, 3.33%), Renmin Hospital of Wuhan University (13/390, 3.33%), Guangzhou Eighth People's Hospital (11/390, 2.82%), Huoshenshan Hospital (11/390, 2.82%), and Leishenshan Hospital (11/390, 2.82%), **Table 2**.

A cluster analysis was performed for institutions that participated in more than four ICTs. A total of 32 institutions have established cooperative relations and formed six clusters (**Figure 3**). The largest cooperative team consisted of nine hospitals and research institutions. The smallest team only included three institutions. There was relatively more cooperation between institutions within the team. However, collaboration between different teams was sparse.

Interventions

There were various types of interventions. Commonly used western medicines included Lopinavir/Ritonavir (34 times), Mesenchymal stem cells (21 times), Interferon α (18 times), Chloroquine phosphate (15 times), Favipiravir (14 times), SARS-COV-2 inactivated/convalescent plasma (10 times), Arbidol (10 times), Thymosin (eight times), Tocilizumab (seven times), Hydroxychloroquine sulfate (six times), and Arbidol hydrochloride (six times). Other western medicines were used less than six times, such as Azvudine, Hydroxychloroquine, Ritonavir, and Remdesivir. A total of 125/390 (32.05%) ICTs focused on traditional Chinese medicine or integrated traditional Chinese and Western medicine, of which 55/390 (14.10%) ICTs mentioned traditional Chinese medicine treatment, traditional Chinese medicine syndrome differentiation treatment, or integrated traditional Chinese and western medicine treatment, but they did not provide specific names of medicine. Among ICTs that provided the specific Chinese medicine, drugs that appeared more than once included Honeysuckle decoction/oral liquid (four times), Xiyanping injection (four times), Shuanghuanglian oral liquid (three times), Lianhua Qingwen capsules/granules

TABLE 2 | Institutions contributed to the registration of COVID-19 ICTs (>5) [N (%)].

Rank	Institutions	N (%)
1	Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology	30 (7.69%)
2	Zhongnan Hospital of Wuhan University	18 (4.62%)
3	Wuhan Jinyintan Hospital	18 (4.62%)
4	Shanghai Public Health Clinical Center	17 (4.36%)
5	Union Hospital, Tongji Medical College, Huazhong University of Science and Technology	14 (3.59%)
6	The First Affiliated Hospital of Guangzhou Medical University	13 (3.33%)
7	Renmin Hospital of Wuhan University	13 (3.33%)
8	Guangzhou Eighth People's Hospital	11 (2.82%)
9	Huoshenshan Hospital	11 (2.82%)
10	Leishenshan Hospital	11 (2.82%)
11	Hubei Integrated Traditional Chinese and Western Medicine Hospital	10 (2.56%)
12	Hubei Provincial Hospital of Traditional Chinese Medicine	10 (2.56%)
13	The First Affiliated Hospital of Zhejiang University School of Medicine	10 (2.56%)
14	Hospital of Chengdu University of Traditional Chinese Medicine	8 (2.05%)
15	Huangshi Hospital of Traditional Chinese Medicine	8 (2.05%)
16	The First Affiliated Hospital of Nanchang University	8 (2.05%)
17	The First Affiliated Hospital of Wenzhou Medical University	8 (2.05%)
18	Beijing You'an Hospital, Capital Medical University	7 (1.79%)
19	West China Hospital of Sichuan University	7 (1.79%)
20	Wuhan Third People's Hospital	7 (1.79%)
21	Wuhan Pulmonary Hospital	7 (1.79%)
22	The First Hospital of Peking University	6 (1.54%)
23	Ruijin Hospital Affiliated to Shanghai Jiaotong University School of Medicine	6 (1.54%)
24	Longhua Hospital Affiliated to Shanghai University of Traditional Chinese Medicine	6 (1.54%)
25	The Third People's Hospital of Shenzhen	6 (1.54%)
26	The Fifth Affiliated Hospital of Sun Yat-Sen University	6 (1.54%)

(two times), Babaodan (two times), Maxingshigan decoction (two times), Qingfeipaidu decoction (two times), Tanreqing capsule/injection (two times), Xuebijing injection (two times), and Yinhu Qingwen decoction/granules (two times). The remaining Chinese medicines appeared only once, such as Baidu Duan Fang, Bufeihuoxue capsule, Shenqi Fuzheng injection, Fuzheng Huayu tablets, Shenlingbaizhu powder, and Reduning injection.

Outcome Measures

Primary Outcome Measures

The number of ICTs with one primary outcome measure was the largest, with 193/390 (49.49%) ICTs. A total of 74/390 (18.97%) ICTs had two primary outcome measures, 47/390 (12.05%) ICTs with three primary outcome measures, and 6/390 (1.54%) ICTs with more than 12 primary outcome measures (**Figure 4**). **Figure 5** shows the primary outcome measures with frequencies greater than two times, which includes 51 outcomes on the map. As shown in **Figure 5** and **Table 3**, chest/lung CT (53/390, 13.59%) was the most commonly used primary outcome measure, followed by the time of viral nucleic acid turning negative (40/390, 10.26%), clinical recovery time (35/390, 8.97%), incidence of adverse events (30/390, 7.69%), clinical improvement time (23/390, 5.90%), clinical symptoms improvement (23/390, 5.90%), mortality (19/390, 4.87%), rate of viral nucleic acid turning negative (19/390, 4.87%), hospital stay (16/390, 4.10%), and blood routine (15/390, 3.85%).

Secondary Outcome Measures

Of the 390 ICTs, 279 (71.54%) ICTs have secondary outcomes. **Figure 6** shows the secondary outcome measures with frequencies greater than two times, which includes 49 outcomes on the map. Hospital stay (33/390, 8.46%) was the most commonly used secondary outcome measure, followed by all-cause mortality (30/390, 7.69%), incidence of adverse events (25/390, 6.41%), time of viral nucleic acid turning negative (22/390, 5.64%), rate of progression to severe (20/390, 5.13%), mortality (18/390, 4.62%), chest/lung CT (17/390, 4.36%), C-reactive protein (17/390, 4.36%), clinical improvement time (16/390, 4.10%), and incidence of serious adverse events (16/390, 4.10%), **Table 4**.

DISCUSSION

A total of 29 provinces from China contributed to the registration of COVID-19 ICTs, of which 55.17% provinces participated in $<$ 10 ICTs, while Hubei province participated in 160 ICTs, indicating that ICTs registrations were mainly concentrated in a few provinces. Through the network analysis of provinces,

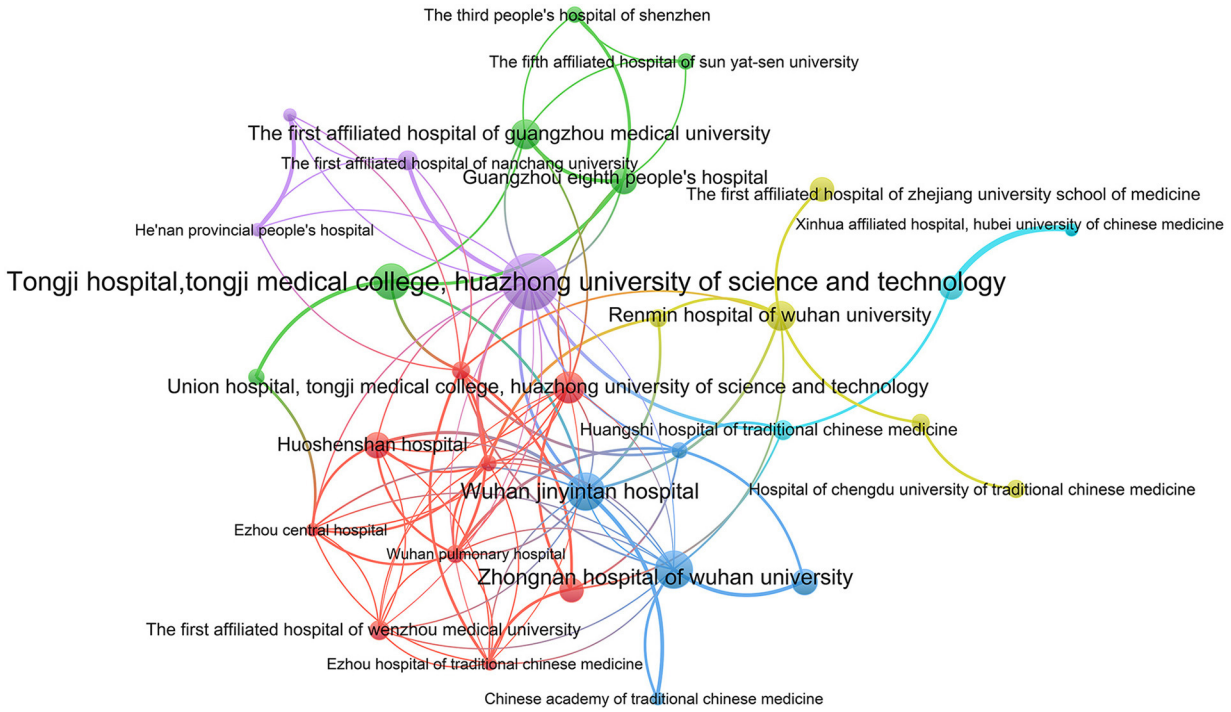


FIGURE 3 | The network map of institutions for registered ICTs of COVID-19.

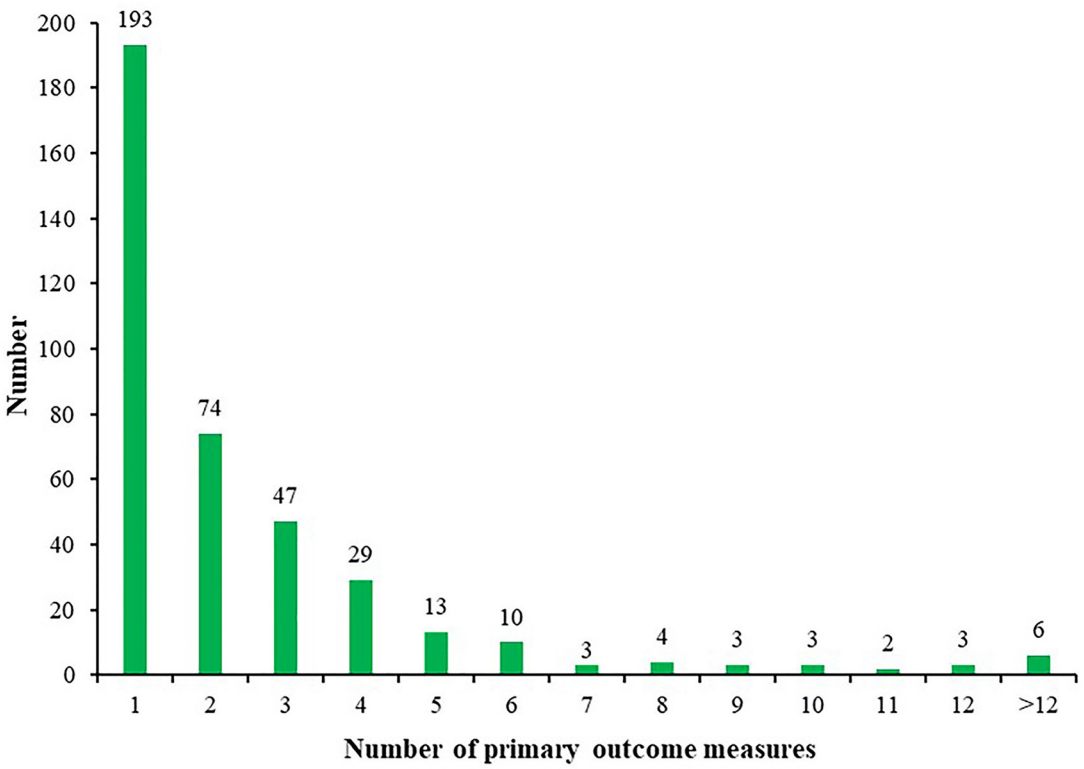


FIGURE 4 | Distribution of the number of primary outcome measures for individual ICT of COVID-19.

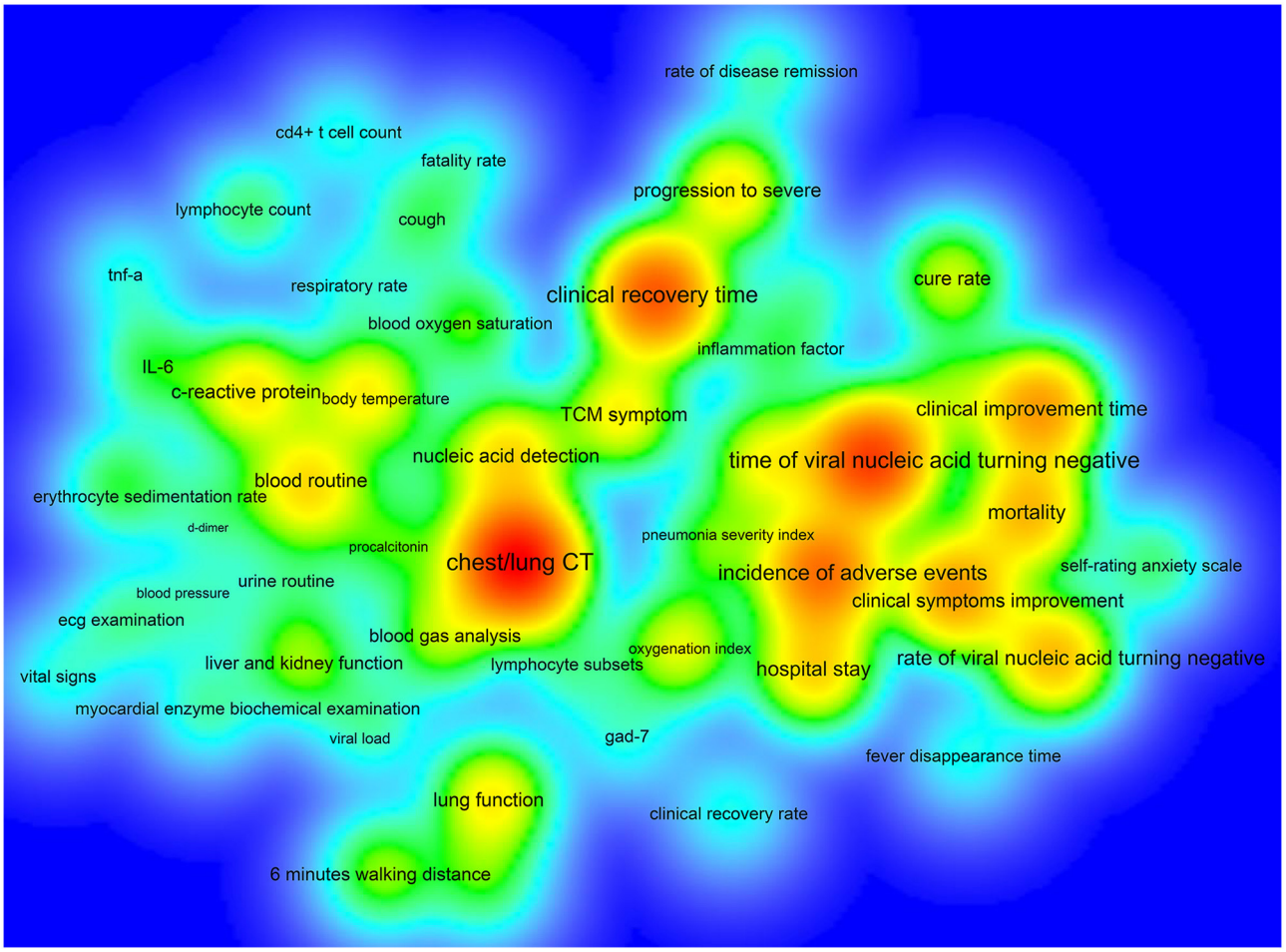


FIGURE 5 | The density map of high-frequency primary outcome measures for registered ICTs of COVID-19.

we found that Hubei and Beijing had more collaborations with other provinces, but the collaborations between the remaining provinces were not close. A total of 430 institutions participated in the registration of COVID-19 ICTs, but only 26 institutions participated in the registration of more than five ICTs, and 80.93% of the institutions contributed to only one or two ICTs. The productive institutions formed six cooperative teams and the number of institutions within the teams did not exceed nine. The cooperation between institutions within each team was relatively close, but cooperation between different teams was sparse. Therefore, future researchers should strengthen more comprehensive and extensive cooperation between different provinces and different regions. Through the analysis of the sample size, we found that the sample size of 26.67% ICTs was lower than 50. Some ICTs only included 10 patients, which were inadequate. 12.82% of ICTs had a sample size $>$ 300, with the maximum sample size up to 20,000, but the sample size of some ICTs was too large to be performed in just one institution, as the sample size far exceeds the total number of patients in their region. However, they did not carry out cross-institutional and cross-regional cooperation. Besides, patients before the trial should be ruled out, which shows that it is difficult to complete the trial according to the research protocol. This also shows that it is necessary to strengthen cooperation and exchanges and carry out multi-center research.

In clinical trials, many strategies have been tried to treat COVID-19. Although there is no specific drug for COVID-19 (15), the drug used in clinical trials should also be carefully chosen to avoid additional damage to the patient's health. The commonly studied western medicines included Lopinavir/Ritonavir, Mesenchymal stem cells, Interferon α, Chloroquine phosphate, Hydroxychloroquine sulfate, Favipiravir, and Arbidol. However, the sample sizes of many trials were insufficient, and the usage, dosage, and treatment course of drugs were unclear, which may lead to a lack of credibility in the results of the research. Therefore, future researchers should conduct large-scale, multi-center clinical trials, rather than repeating trials for an intervention, to avoid wasting resources. Of the 125 ICTs concerned with traditional Chinese medicine or integrated traditional Chinese and Western medicine, about 45.00% of the trials did not provide specific names and usages of traditional Chinese

TABLE 3 | The top 20 primary outcome measures in terms of frequency [N (%)].

Rank	Primary outcome measures	N (%)	Rank	Primary outcome measures	N (%)
1	Chest/lung CT	53 (13.59%)	11	Nucleic acid detection	15 (3.85%)
2	Time of viral nucleic acid turning negative	40 (10.26%)	12	C-reactive protein	14 (3.59%)
3	Clinical recovery time	35 (8.97%)	13	Rate of progression to severe	14 (3.59%)
4	Incidence of adverse events	30 (7.69%)	14	Body temperature	13 (3.33%)
5	Clinical improvement time	23 (5.90%)	15	Lung function	13 (3.33%)
6	Clinical symptoms improvement	23 (5.90%)	16	TCM symptom	13 (3.33%)
7	Mortality	19 (4.87%)	17	Antipyretic time	12 (3.08%)
8	Rate of viral nucleic acid turning negative	19 (4.87%)	18	Oxygenation index	11 (2.82%)
9	Hospital stay	16 (4.10%)	19	Cure rate	10 (2.56%)
10	Blood routine	15 (3.85%)	20	Blood gas analysis	9 (2.31%)

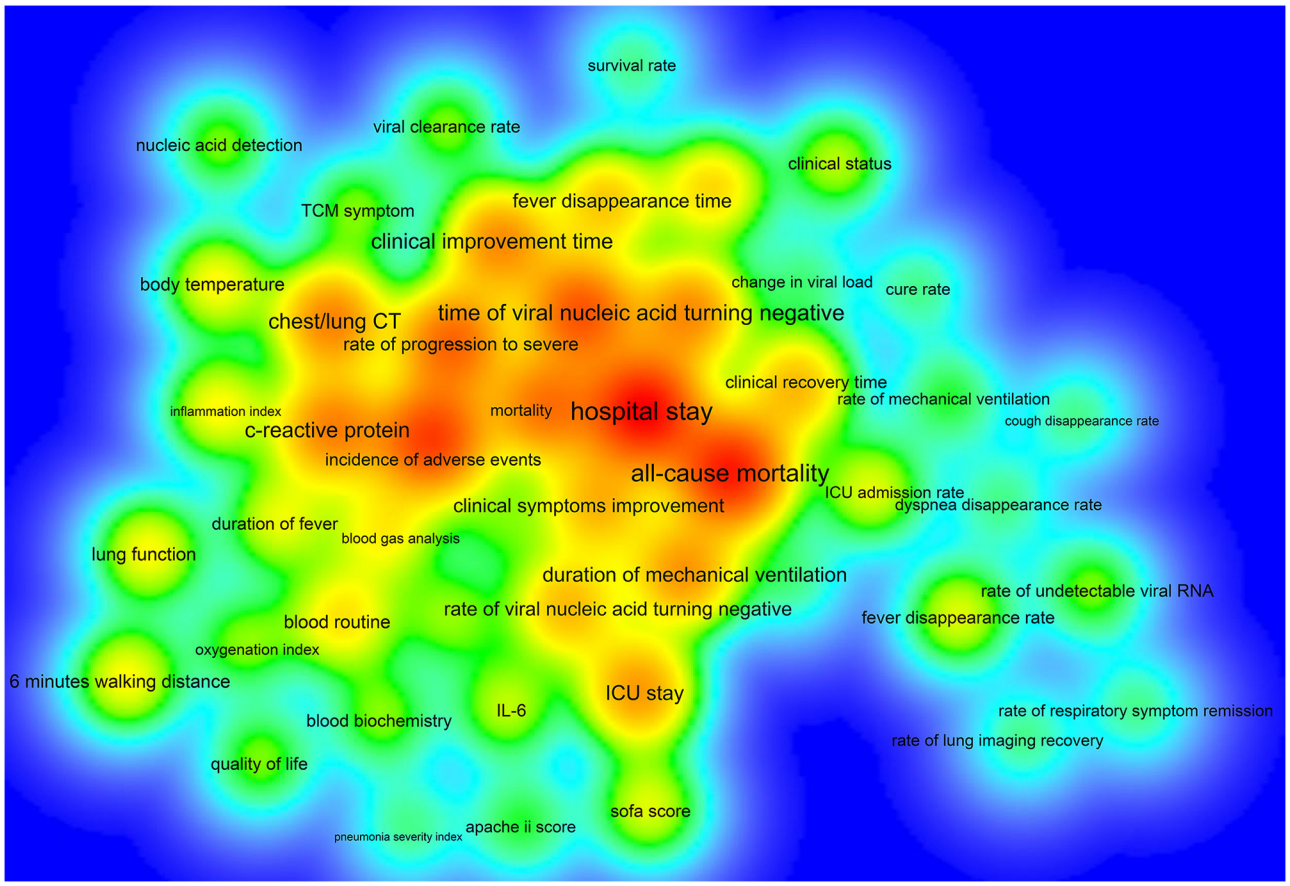


FIGURE 6 | The density map of high-frequency secondary outcome measures for registered ICTs of COVID-19.

medicine. Besides, the most commonly used control was the usual treatment, but most ICTs did not provide specific content of the usual treatment. Future trial registers and reviewers of registry platforms should pay more attention to these aspects to promote the registration of COVID-19 clinical trials more standardized.

Some ICTs only adopted one primary outcome measure, and some ICTs had more than 12 primary outcome measures, which indicated that there was a considerable difference in the number of primary outcomes. Chest/lung CT, time of viral nucleic acid turning negative, the incidence of adverse events, clinical improvement time, mortality, and hospital stay were among the top 10 primary outcomes, as well as among the top ten secondary outcomes, indicating that these six outcome measures were key outcomes in this field. Future researchers can use these measures when conducting COVID-19 clinical

TABLE 4 | The top 20 secondary outcome measures in terms of frequency [N (%)].

Rank	Secondary outcome measures	N (%)	Rank	Secondary outcome measures	N (%)
1	Hospital stay	33 (8.46%)	11	Duration of mechanical ventilation	15 (3.85%)
2	All-cause mortality	30 (7.69%)	12	ICU stay	15 (3.85%)
3	Incidence of adverse events	25 (6.41%)	13	Clinical recovery time	12 (3.08%)
4	Time of viral nucleic acid turning negative	22 (5.64%)	14	Clinical symptoms improvement	12 (3.08%)
5	Rate of progression to severe	20 (5.13%)	15	Rate of viral nucleic acid turning negative	12 (3.08%)
6	Mortality	18 (4.62%)	16	Fever disappearance time	11 (2.82%)
7	Chest/lung CT	17 (4.36%)	17	Duration of supplemental oxygenation	10 (2.56%)
8	C-reactive protein	17 (4.36%)	18	Blood routine	9 (2.31%)
9	Clinical improvement time	16 (4.10%)	19	Blood gas analysis	8 (2.05%)
10	Incidence of serious adverse events	16 (4.10%)	20	Body temperature	8 (2.05%)

trials. This study found that there are some problems with the outcome measures: (1) there were too many types of indicators and lack of main outcome measures, which added difficulties to the development of systematic reviews and guidelines; (2) the expression of outcome measures was not standardized, and there were multiple expression terms for the same measure; (3) the definitions of outcome measures were not clear, and many outcome measures were ambiguous; (4) most ICTs did not clarify the time of follow-up and the measurement time of the outcomes; (5) the selected outcome measures cannot fully reflect the expected research results; (6) regarding outcomes that need to be measured, most ICTs did not provide measurement tools; and (7), considering ICTs that focused on the traditional Chinese medicine and integrated traditional Chinese and Western medicine, there was a lack of outcome measures with characteristics of traditional Chinese medicine. These shortcomings need to be further improved for future clinical trials of COVID-19.

We conducted a comprehensive analysis of the registered ICTs of COVID-19 conducted in China using the bibliometric analysis method and presented collaborations of provinces and institutions, and the distribution of outcome measures by using visual network maps and density maps. However, this study also has some limitations. Firstly, only ICTs from China were included, and many clinical trials will be registered in the future, which cannot fully reflect the status of all clinical trials and may not apply to ICTs in other countries. Secondly, since some institutions, interventions, and outcomes have different expressions, although we have standardized them, bias may still exist. Thirdly, some registered ICTs may not provide all participating institutions, resulting in the results of this study may differ from the actual situation. Finally, since this study was based on data of registered ICTs, we did not explore the effectiveness of the interventions and outcome measures. Further studies are needed to assess whether the registered ICTs have been completed and whether the interventions and outcome measures studied are effective.

During the COVID-19 pandemic, we are very pleased that scholars from all over the world are actively conducting clinical trials to explore effective drugs for the treatment of COVID-19. However, our study found that the registered ICTs had many defects in methods and results. Therefore, future researchers should optimize the methods of these trials and ensure the transparency of their methods to produce high-quality evidence. Otherwise, it will not only result in a waste of resources and property, but more importantly, mislead the measures to deal with COVID-19 and delay treatment for patients. Furthermore, researchers should facilitate the completion of these clinical trials and translate the results of these trials into practices and policies.

CONCLUSIONS

The number of daily registrations for ICTs of COVID-19 fluctuated significantly. Hubei, Shanghai, and Beijing are the top three productive provinces. Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Zhongnan Hospital of Wuhan University, and Wuhan Jinyintan Hospital are the top three productive institutions. Collaborations between provinces and institutions were not close enough. More comprehensive and extensive collaborations between different provinces and different regions should be further strengthened. The identified deficiencies in interventions and outcome measures should be given more attention by future researchers of COVID-19.

AUTHOR CONTRIBUTIONS

YG and JT planned and designed the study. YG, KY, ML, YC, and SS participated in the literature search and data collection. YG, KY, ML, and FY analyzed the data. YG and JT drafted the manuscript. YG, FY, and JT revised the manuscript. All authors read and approved the final manuscript.

FUNDING

This work was supported by the Emergency Research Project of Key Laboratory of Evidence-based Medicine and Knowledge Translation of Gansu Province (Grant no. GSEBMKT-2020YJ01) and Tianjin University of

Traditional Chinese Medicine Science and Technology Project (Grant no. TUTCM001).

ACKNOWLEDGMENTS

The authors thank all investigators and supporters involved in this study.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2020.554247/full#supplementary-material

Figure S1 | The flowchart of the screening process.

REFERENCES

1. Guan WJ, Ni ZY, Hu Y, Liang WH, Ou CQ, He JX, et al. Clinical characteristics of coronavirus disease 2019 in China. *N Engl J Med*. (2020) 382:1708–20. doi: 10.1056/NEJMoa2002032
2. Chen T, Wu D, Chen H, Yan W, Yang D, Chen G, et al. Clinical characteristics of 113 deceased patients with coronavirus disease 2019: retrospective study. *BMJ*. (2020) 368:m1091. doi: 10.1136/bmj.m1091
3. Li Q, Guan X, Wu P, Wang X, Zhou L, Tong Y, et al. Early transmission dynamics in Wuhan, China, of novel coronavirus-infected pneumonia. *N Engl J Med*. (2020) 382:1199–207. doi: 10.1056/NEJMoa2001316
4. Gautret P, Lagier JC, Parola P, Hoang VT, Meddeb L, Sevestre J, et al. Clinical and microbiological effect of a combination of hydroxychloroquine and azithromycin in 80 COVID-19 patients with at least a six-day follow up: a pilot observational study. *Travel Med Infect Dis*. (2020) 34:101663. doi: 10.1016/j.tmaid.2020.101663
5. Zhu N, Zhang D, Wang W, Li X, Yang B, Song J, et al. A novel coronavirus from patients with pneumonia in China, 2019. *N Engl J Med*. (2020) 382:727–33. doi: 10.1056/NEJMoa2001017
6. Wu JT, Leung K, Leung GM. Nowcasting and forecasting the potential domestic and international spread of the 2019-nCoV outbreak originating in Wuhan, China: a modelling study. *Lancet*. (2020) 395:689–97. doi: 10.1016/S0140-6736(20)30260-9
7. WHO. *Director-General's Opening Remarks at the Media Briefing on COVID-19: 11 March 2020*. (2020) Available online at: https://www.who.int/dg/speeches/detail/who-director-general-s-opening-remarks-at-the-media-briefing-on-covid-19---11-march-2020 (accessed April 8, 2020).
8. World Health Organization. *Coronavirus Disease (COVID-2019) Situation Reports*. Available online at: https://www.who.int/emergencies/diseases/novel-coronavirus-2019/situation-reports (accessed July 16, 2020).
9. Zhang Q, Wang Y, Qi C, Shen L, Li J. Clinical trial analysis of 2019-nCoV therapy registered in China. *J Med Virol*. (2020) 92:540–5. doi: 10.1002/jmv.25733
10. Wang H, Jin XY, Pang B, Liu CX, Zheng WK, Yang FW, et al. [Analysis on clinical study protocols of traditional Chinese medicine for coronavirus disease 2019]. *Zhongguo Zhong Yao Za Zhi*. (2020) 45:1232–41. doi: 10.19540/j.cnki.cjcmm.20200220.501
11. Gao Y, Ge L, Shi S, Sun Y, Liu M, Wang B, et al. Global trends and future prospects of e-waste research: a bibliometric analysis. *Environ Sci Pollut Res Int*. (2019) 26:17809–20. doi: 10.1007/s11356-019-05071-8
12. Gao Y, Shi S, Ma W, Chen J, Cai Y, Ge L, et al. Bibliometric analysis of global research on PD-1 and PD-L1 in the field of cancer. *Int Immunopharmacol*. (2019) 72:374–84. doi: 10.1016/j.intimp.2019.03.045
13. Liu M, Gao Y, Yuan Y, Shi S, Yang K, Wu J, et al. Global hotspots and future prospects of chimeric antigen receptor T-cell therapy in cancer research: a bibliometric analysis. *Future Oncol*. (2019) 16:597–612. doi: 10.2217/fon-2019-0810
14. Liang YD, Li Y, Zhao J, Wang XY, Zhu HZ, Chen XH. Study of acupuncture for low back pain in recent 20 years: a bibliometric analysis via CiteSpace. *J Pain Res*. (2017) 10:951–64. doi: 10.2147/JPR.S132808
15. Tu YF, Chien CS, Yarmishyn AA, Lin YY, Luo YH, Lin YT, et al. A review of SARS-CoV-2 and the ongoing clinical trials. *Int J Mol Sci*. (2020) 21:2657. doi: 10.3390/ijms21072657

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Medicine

OPINION
published: 02 September 2020
doi: 10.3389/fmed.2020.00569

The Rapid Coronavirus Antibody Test: Can We Improve Accuracy?

Ina P. Pavlova[1]*, *Sujit S. Nair*[1,2], *Natasha Kyprianou*[1,2,3,4] and *Ash K. Tewari*[1,2]

[1] Department of Urology, Icahn School of Medicine at Mount Sinai, New York, NY, United States, [2] The Tisch Cancer Institute, Icahn School of Medicine at Mount Sinai, New York, NY, United States, [3] Department of Oncological Sciences, Icahn School of Medicine at Mount Sinai, New York, NY, United States, [4] Department of Pathology, Icahn School of Medicine at Mount Sinai, New York, NY, United States

Keywords: SARS-CoV-2 antibodies, neutralizing antibodies, rapid tests, lateral flow assays, paper based analytical devices

OPEN ACCESS

Edited by:
Zisis Kozlakidis, International Agency for Research on Cancer (IARC), France

Reviewed by:
Tarek A. Ahmad, Bibliotheca Alexandrina, Egypt
Mengmeng Li, Chinese Academy of Medical Sciences and Peking Union Medical College, China

***Correspondence:**
Ina P. Pavlova
Ina.Pavlova@mountsinai.org

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: *01 June 2020*
Accepted: *11 August 2020*
Published: *02 September 2020*

Citation:
Pavlova IP, Nair SS, Kyprianou N and Tewari AK (2020) The Rapid Coronavirus Antibody Test: Can We Improve Accuracy? Front. Med. 7:569. doi: 10.3389/fmed.2020.00569

INTRODUCTION

We are at a critical stage in managing the response to the COVID-19 outbreak, which requires widespread access to fast and accurate testing. While PCR testing has been the backbone for COVID-19 diagnosis, now there is an urgent need for surveillance of at-risk asymptomatic populations. Antibody tests check for an antibody response to SARS-CoV-2 infection and are used to determine infection and case fatality rates, or potential immunity in recovered patients and in vaccine studies. Effective laboratory SARS-CoV-2 antibody technologies have been developed, and some were validated by the FDA to have Sensitivity (Se) and Specificity (Sp) as high as 99–100%[1]. For example, an IgG two-step ELISA test measures IgG responses to the recombinant receptor binding domain (RBD) of the SARS-CoV-2 spike protein (1). Positive samples are confirmed in a second step that measures IgG response to the whole spike protein (1), resulting in a 100% Sp (with 92.5% Se)[1]. However, while accurate, laboratory technologies are slow and rely on expensive equipment.

Rapid (minutes vs. hours) and instrument-free SARS-CoV-2 assays are commercially available, and some are already being used in surveillance studies. Debates about the recently reported infection rates in NYC (21.1% as of 04/23/20[2]), or in Santa Clara, CA [2.45% (2)], have raised questions regarding whether antibody testing is sufficiently accurate to guide medical or policy decisions. Recently, the COVID-19 Testing Project validated 10 rapid commercial tests in a head-to-head comparison with samples from 80 SARS-CoV-2 RT-PCR-positive, 108 pre-COVID-19 negative, and 52 recently negative patients (3). Many rapid tests performed worse than their manufacturer's specifications, raising questions about their quality and stability. Moreover, while high specificity is crucial for testing low prevalence population (estimated COVID-19 prevalence is only ∼5%), only three out of 10 rapid tests had a Sp of >99%, while maintaining >90% Se (at >16 days after onset of symptoms) (3). More recently, the FDA started their own validation of 13 EUA approved antibody tests and found that only one of the validated rapid tests has a > 99% Sp (with a 95% Se)[1]. Introducing more stringent FDA criteria has driven the need for highly accurate rapid tests[3]. Here we summarize some of the limitations of rapid COVID-19 antibody tests and suggested ways for improvement.

[1]https://www.fda.gov/medical-devices/emergency-situations-medical-devices/eua-authorized-serology-test-performance
[2]https://publish.twitter.com/?query=https%3A%2F%2Ftwitter.com%2FNYGovCuomo%2Fstatus%2F1253353968278876171&widget=Tweet
[3]https://www.fda.gov/news-events/fda-voices/insight-fdas-revised-policy-antibody-tests-prioritizing-access-and-accuracy

ISOTYPE-SPECIFIC (IgM/IgG) DETECTION

After SARS-CoV-2 infection, IgM or IgG antibodies appear in the patient's blood that are specific for viral antigens to the spike glycoprotein such as the S1, S2 subunits, the receptor binding domain (RBD) or the nucleocapsid (N) protein (1). First, IgM becomes detectable within a few days and lasts several weeks after infection, followed by IgG detection. Currently, all rapid SARS-CoV-2 antibody tests rely on the ability of recombinant proteins of RBD, S1, S2, or the N domain of the SARS-CoV-2 spike protein to capture IgM or IgG antibodies in the patient's blood[3] (4, 5). This isotype-specific detection (IgM or IgG) is time dependent; high sensitivity rates are achieved only at 3 weeks from symptom onset (3). For example, the COVID-19 Testing Project (3) showed that overall sensitivity of all validated rapid tests reached >80% Se only at >20 days of symptom onset (maintaining 95% Sp). None of the tests showed >80% Se at 6–10 days of symptom onset and only half showed >80% Se at 11–15 days of symptom onset.

Moreover, these validated rapid tests tend to have a higher Se for patients admitted to ICU compared to patients with milder disease (3). Recent clinical studies of antibody responses in patients with COVID-19 have associated higher IgG and IgM titers with worse disease outcome at all time points following the onset of symptoms (6), or with worse clinical readouts and older age (7). These findings suggest that rapid assay kits may favor the detection of higher IgG and IgM titers, and therefore perform better in more severe disease. In addition, while a growing number of studies report that SARs-CoV-2 antibodies are best detectable in infected people 3–4 weeks after symptom onset (8, 9), the antibody levels are lower and may have different kinetics in people with milder symptoms (10) and are is still largely unknown in asymptomatic people (9). This suggests that timing and choice of assays may have to be optimized depending on the populations to be tested. On the other hand, a study characterizing the neutralizing antibodies (Nabs) response in a cohort of COVID-19 recovered patients with mild symptoms, found a persistent Nabs response in 70% of recovered patients, with SARS-CoV-2-specific Nabs detected as early as 10–15 days after disease onset with kinetics aligned to that of binding antibodies (11). This suggests that Nabs detection could be performed in parallel to rapid isotype specific IgG and IgM detection to provide information about the functionality of the antibody repose and potential protection.

Rapid antibody tests capture binding IgG and IgM antibodies but not necessarily neutralizing antibodies (4, 5). Binding antibodies do not have the same neutralizing abilities or high affinity to the spike protein antigens as neutralizing antibodies (12). Recently, a SARS-CoV-2 surrogate virus neutralization test (sVNT) was developed that detects total neutralizing antibodies in an isotype-independent manner (13). This test utilizes the high-affinity interaction between the receptor binding domain (RBD) protein from the viral spike (S) protein and the host cell receptor ACE2 (hACE2) (14). Neutralizing antibodies inhibit this interaction by binding to the RBD protein prior to the virus-host interaction (12, 13). The sVNT test mimics this process by utilizing recombinant ACE2 and RBD proteins and detecting the % antibody-mediated inhibition (13). This test was validated to have 100% Sp (while maintaining 96% Se) in two patient cohorts. Moreover, its authors report superior sensitivity for low IgM/IgG titers compared to isotype-specific capture ELISA (13), suggesting that it can be used for testing in populations with lower levels of antibodies such as mildly symptomatic populations. However, currently its sensitivity is not validated by other studies and it is not yet adapted for rapid detection platforms.

LATERAL FLOW DETECTION

Rapid SARS-CoV-2 antibody assays utilize lateral flow detection. Lateral flow tests are performed on a low-cost nitrocellulose strip which has assay reagents dried on the test zone. The target analyte diffuses from the sample deposition pad to the test zone by capillary action, and readout of the test zone is based on colorimetric detection (with gold nanoparticles conjugated to a detection antibody or recombinant protein), which eliminates the need for laboratory instruments. However, lateral flow tests are prone to variability due to many factors, including quality of the nitrocellulose and recombinant proteins, and their stability after drying. Moreover, simple lateral flow designs cannot perform multistep, sequential processes. Many laboratory assays rely on sequential washing and signal amplification steps for improved specificity and sensitivity. To enhance lateral flow designs, two-dimensional paper devices have been previously developed that allow for the timely delivery of multiple reagents to the test zone (15–17). These devices utilize capillary action and dried reagents, but their design incorporates additional compartments with detection, signal amplification or wash reagents so that fixed reagent volumes are delivered to the test zone in a sequential and controlled way. Such two-dimensional paper devices have previously been used successfully for the detection of antibodies against HPV and malaria (15–17), but not against SARS-CoV-2.

NEW TESTING APPROACHES

One approach to improve the accuracy of rapid SARS-CoV-2 antibody tests is to adapt isotype independent assays, such as the sVNT test on lateral flow formats. Most current lateral flow tests have separate test zones for IgM and IgG detection, requiring two sets of capture and detection reagents (**Figure 1A**). However, a lateral flow sVNT assay would have only one test zone, simplifying reagent requirements (**Figure 1B**). We also suggest that a lateral flow sVNT test will have improved sensitivity, because it detects neutralizing antibodies with higher affinity to the recombinant RBD antigen than binding antibodies, optimizing capturing of the target analyte on the test strip. Further improvement could be achieved by integrating with a multi-step paper-based device (**Figure 1C**). This design allows for sequential delivery of a wash prior to the detection step

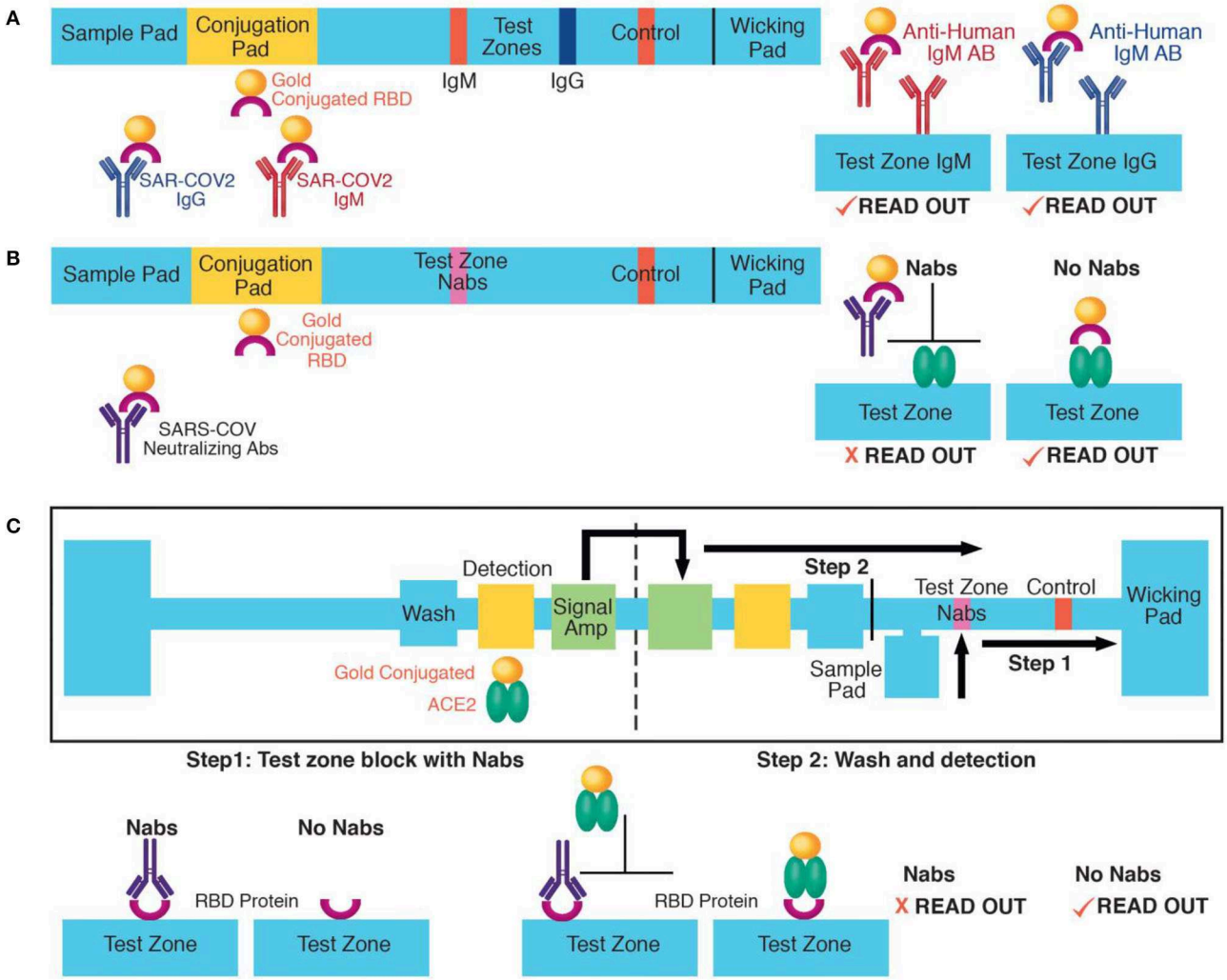


FIGURE 1 | Approaches for rapid SARS-COV-2 antibody detection: **(A)** Example of IgM and IgG lateral flow detection. Antibodies move from the sample to the conjugation pad by capillary action where they bind dried recombinant RBD proteins conjugated to gold nanoparticles. Next, they are captured on the IgM or the IgG test zone. Aggregated nanoparticles at the test zones results in colorimetric readout. **(B)** A lateral flow detection of neutralizing antibodies (Nabs). A proposed application of the sVNT assay (13). Nabs move from the sample pad to the test zone by capillary action. At the conjugation pad, they bind to dried recombinant RBD proteins causing their neutralization. At the test zone, neutralized RBD proteins cannot bind to immobilized recombinant ACE2 and are washed out. In the absence of neutralizing antibodies, RBD proteins bind to ACE2 proteins at the test zone, causing colorimetric readout. **(C)** A multi-step, paper-based test for neutralizing antibodies. In step one, Nabs move from the sample pad to the test zone by capillary action and block recombinant RBD proteins immobilized at the test zone. **(C)** The left part of the device has a wash, detection (with dried recombinant ACE2 proteins conjugated to gold nanoparticles) and signal amplification pad. In step two, the device is folded to initiate to sequential delivery of a wash, detection, and signal amplification volume to the test zone. In the absence of neutralizing antibodies, the ACE2-gold nanoparticle complex binds to the test zone. The paper-based device schematic was adapted from (17).

(reducing false positives); and a final signal amplification step (optimizing sensitivity), while keeping a user-friendly, instrument-free, and disposable platform. In addition, testing a population with low prevalence of infection is challenging because even a highly specific assay can result in many false positive results. Therefore, an approach for decreasing false positives is to add confirmatory steps to lateral flow or paper-based devices, such as multiple test zones on the same test strip allowing binding to different viral epitopes (e.g., recombinant RBD test zone with confirmatory zones with the S1, S2, or N domains).

DISCUSSION

Results from SARS-CoV-2 testing influence the effective management of the current health crisis. Here we have outlined several factors that limit the accuracy of currently used rapid serological tests. First, most rapid tests utilize lateral flow detection with one-step delivery of the target analyte and detection reagents, which we argue limits their accuracy. Previously, multi-step paper-based platforms with time- and volume-controlled delivery of the target analyte and detection reagent have been validated for the detection of infectious

diseases (15, 17). Exploiting such platforms for the detection of SARS-CoV-2 antibodies allows incorporating wash and signal amplification steps and sequential reagent delivery, currently lacking from rapid tests designs. We suggest that these additions will improve both sensitivity (due to signal amplification) as well as specificity (due to the wash between the sample and detection reagent delivery), while still maintaining a paper-based, disposable and cost effective platform. In addition, we argue that current rapid SARS-CoV-2 kits (based on isotype-specific IgM/IgG assays) favor detection of higher antibody titers. Specifically, since patients with more severe disease have higher titers (6, 7), we argue that these kits may have higher false negative rates when testing populations with mild disease as compared to those with severe symptoms and disease. Assays with better sensitivity for low titers such as such as the recently developed sVNT test for SARS-CoV-2 neutralizing antibodies (13) need to be applied on rapid detection platforms. Here we suggest that approaches for combining new antibody-based assays with multi-step, paper-based devices should be further exploited to improve the accuracy of current rapid SARS-CoV-2 testing. Formulation of these devices is straightforward and scalable; it requires only simple, low cost materials, such as nitrocellulose and glass fiber filters, and a laser cutter (17), as well as high quality recombinant SARS-COV-2 proteins, that are already commercially available (GenScript, Piscataway, NJ). Therefore, the proposed approaches will potentially provide a technology that is rapid and accurate, as well as scalable and low-cost, making it an attractive solution for mass screening of large populations.

Finally, SARS-CoV-2 antibody tests, even when highly accurate, would detect infection at best 2 to 3 weeks after symptom onset, which raises questions about how to optimize testing approaches for mildly or asymptomatic populations. For example, a study on the immune response of patients with mild disease report that IgG antibodies titers peaked around 24 days from symptom onset, suggesting that antibody testing should be done at least 3 to 4 weeks after symptom onset (11). This study also reports that in a cohort of people with suspected disease, only 36% of cases had a positive antibody test result. The authors suggest that this is partially due to insufficient time testing for mounting an antibody response, which emphasizes that improving detection of SARS-CoV-2 infection requires expanded viral load as well as antibody response testing. In line with these findings, we suggest that an optimized surveillance approach for mildly or asymptomatic populations could involve rapid testing for antibodies, as well as viral load. While PCR testing for viral load requires expensive laboratory equipment, many rapid and isothermal nucleic acid amplification approaches have been already developed for point of care applications. Moreover, recently the FDA approved the first SARS-CoV-2 antigen test that detects virus particles without needing PCR[4]. Therefore, one way to optimize screening of mildly or asymptomatic populations is to develop one integrated rapid paper-based test to detect both SARS-CoV-2 antibody status and virus load.

AUTHOR CONTRIBUTIONS

IP contributed to the conception, research, and writing and editing of manuscript. SN, NK, and AT were involved in the research and writing and editing of the manuscript. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

The authors wish to acknowledge funding from the Deane Prostate Health and ISMMS.

[4]https://www.fda.gov/news-events/press-announcements/coronavirus-covid-19-update-fda-authorizes-first-antigen-test-help-rapid-detection-virus-causes

REFERENCES

1. Amanat F, Stadlbauer D, Strohmeier S, Nguyen THO, Chromikova V, McMahon M, et al. A serological assay to detect SARS-CoV-2 seroconversion in humans. *Nat Med*. (2020) 26, 1033–36. doi: 10.1038/s41591-020-0913-5
2. Bendavid E, Mulaney B, Sood N, Shah S, Ling E, Bromley-Dulfano R, et al. COVID-19 Antibody seroprevalence in Santa Clara County, California. *medRxiv*. (2020). doi: 10.1101/2020.04.14.20062463
3. Whitman JD, Hiatt J, Mowery CT, Shy BR, Yu R, Yamamoto TN, et al. Test performance evaluation of SARS-CoV-2 serological assays. *medRxiv*. (2020). doi: 10.1101/2020.04.25.20074856
4. Tang YW, Schmitz JE, Persing DH, Stratton CW. The laboratory diagnosis of COVID-19 infection: current issues and challenges. *J Clin Microbiol*. (2020) 58:e00512–20. doi: 10.1128/JCM.00512-20
5. Petherick A. Developing antibody tests for SARS-CoV-2. *Lancet*. (2020) 395:1101–2. doi: 10.1016/S0140-6736(20)30788-1
6. Tan W, Lu Y, Zhang J, Wang J, Dan Y, Tan Z, et al. Viral kinetics and antibody responses in patients with COVID-19. *medRxiv*. (2020). doi: 10.1101/ 2020.03.24.20042382
7. Jiang H, Li Y, Zhang H, Wang W, Men D, Yang X, et al. Global profiling of SARS- CoV-2 specific IgG/IgM responses of convalescents using a proteome microarray. *medRxiv*. (2020). doi: 10.1101/2020.03.20.20039495
8. Wajnberg A, Mansour M, Leven E, Bouvier NM, Patel G, Firpo A, et al. Humoral immune response and prolonged PCR positivity in a cohort of 1343 SARS-CoV 2 patients in the New York City region. *medRxiv*. (2020). doi: 10.1101/2020.04.30.20085613
9. Koopmans M, Haagmans B. Assessing the extent of SARS-CoV-2 circulation through serological studies. *Nat Med*. (2020) 26:1171–72. doi: 10.1038/s41591-020-1018-x
10. Long Q, Tang X, Shi Q, Li Q, Deng H, Yuan J, et al. Clinical and immunological assessment of asymptomatic SARS-CoV-2 infections. *Nat Med*. (2020) 26, 1200–04. doi: 10.1038/s41591-020-0965-6
11. Wu F, Wang A, Liu M, Wang Q, Chen J, Xia S, et al. Neutralizing antibody responses to SARS- CoV-2 in a COVID-19 recovered patient cohort and their implications. *medRxiv*. (2020). doi: 10.1101/2020.03.30.20047365
12. Iwasaki A, Yang Y. The potential danger of suboptimal antibody responses in COVID-19. *Nat Rev Immunol*. (2020) 20, 339–41. doi: 10.1038/s41577-020-0321-6
13. Tan CW, Chia WN, Qin X, Liu P, Chen MI, Tiu C, et al. A SARS-CoV-2 surrogate virus neutralization test based on antibody-mediated blockage of ACE2–spike protein–protein interaction. *Nat*

Biotechnol. (2020). doi: 10.1038/s41587-020-0631-z. [Epub ahead of print].
14. Zhou P, Yang XL, Wang XG, Hu B, Zhang L, Zhang W, et al. A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature*. (2020) 579:270–3. doi: 10.1038/s41586-020-2012-7
15. Fridley GE, Le H, Yager P. Highly sensitive immunoassay based on controlled rehydration of patterned reagents in a 2-dimensional paper network. *Anal Chem*. (2014) 86:6447–53. doi: 10.1021/ac500872j
16. Fu E, Liang T, Spicar-Mihalic P, Houghtaling J, Ramachandran S, Yager P. Two-dimensional paper network format that enables simple multistep assays for use in low-resource settings in the context of malaria antigen detection. *Anal Chem*. (2012) 84:4574–9. doi: 10.1021/ac300689s
17. Grant BD, Smith CA, Castle PE, Scheurer ME, Richards-Kortum R. A paper-based immunoassay to determine HPV vaccination status at the point-of-care. *Vaccine*. (2016) 34:5656–63. doi: 10.1016/j.vaccine.2016.09.021

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 02 September 2020
doi: 10.3389/fimmu.2020.02167

Scientific Rationale for a Bottom-Up Approach to Target the Host Response in Order to Try and Reduce the Numbers Presenting With Adult Respiratory Distress Syndrome Associated With COVID-19. Is There a Role for Statins and COX-2 Inhibitors in the Prevention and Early Treatment of the Disease?

Geoffrey Mark Verrall[1,2]*

[1] South Australian Sports Institute, Adelaide, SA, Australia, [2] Sports and Arthritis Clinic, Adelaide, SA, Australia

OPEN ACCESS

Edited by:
Fabio Bagnoli,
GlaxoSmithKline, Italy

Reviewed by:
Paul D. Thompson,
Hartford HealthCare, United States
Ichiro Sakuma,
Hokko Memorial Hospital, Japan

***Correspondence:**
Geoffrey Mark Verrall
geoffrey.verrall@gmail.com

Specialty section:
This article was submitted to Vaccines and Molecular Therapeutics, a section of the journal Frontiers in Immunology

Received: *27 April 2020*
Accepted: *10 August 2020*
Published: *02 September 2020*

Citation:
Verrall GM (2020) Scientific Rationale for a Bottom-Up Approach to Target the Host Response in Order to Try and Reduce the Numbers Presenting With Adult Respiratory Distress Syndrome Associated With COVID-19. Is There a Role for Statins and COX-2 Inhibitors in the Prevention and Early Treatment of the Disease? Front. Immunol. 11:2167. doi: 10.3389/fimmu.2020.02167

The inflammatory response to and the subsequent development of Adult Respiratory Distress Syndrome (ARDS) is considered to underpin COVID-19 pathogenesis. With a developing world catastrophe, we need to examine our known therapeutic stocks, to assess suitability for prevention and/or treatment of this pro-inflammatory virus. Analyzing commonly available and inexpensive immunomodulatory and anti-inflammatory medications to assess their possible effectiveness in improving the host response to COVID-19, this paper recommends the following: (1) optimize current health—cease (reduce) smoking, ensure adequate hypertension and diabetes control, continue exercising; (2) start on an HMG CoA reductase inhibitor "statin" for its immunomodulatory and anti-inflammatory properties, which may reduce the mortality associated with ARDS; and (3) consider using Diclofenac (or other COX-2 inhibition medications) for its anti-inflammatory and virus toxicity properties. For purposes of effectiveness, this needs to be in the early course of the disease (post infection and/or symptom presentation) and given in a high dose. The downsides to these recommended interventions are considered manageable at this stage of the pandemic.

Keywords: COVID-19, COX-2 inhibitors, statins, immunomodulatory, Diclofenac

INTRODUCTION

With an emergency response needed for an emerging viral disease, such as COVID-19, it is unlikely a top-down approach to the development of specific vaccine and drugs will be possible or even effective in the time frame required to meet such a threat. What can be examined is a bottom-up approach, hopefully with some common easily obtainable and inexpensive medications, that can target the host response to the virus (1).

This manuscript involves understanding the mechanism of pathophysiology of disease and previous experiments (mostly laboratory based) to understand how a treatment works and empirical direct observations that the proposed treatments do work.

THE PATHOGENESIS OF SARS-CoV 2 (COVID-19)

Much of our information about COVID-19 comes from the more lethal but less communicable SARS-CoV epidemic. The considered pathogenesis is an inflammatory response of the lung cells that overwhelms the system with a cytokine storm (2). Cytokines are proteins that orchestrate inflammatory response. Risk factors include being older and having hypertension and diabetes. However, the epidemiology and risk factors are not entirely clear with this newly recognized virus.

The entry point for the virus is the Angiotensin Converting Enzyme 2 (ACE2) receptor located on epithelial cells (3). ACE2 receptors are proteins on the surface of many cell types with a high abundance on the Type 2 pneumocytes of the lungs. The spike-like protein of the SARCoV2 binds to ACE2 allowing entry and infection of cells. A host-mediated response then occurs with an induction of the inflammatory (Cyclo-Oxygenase Enzyme-2) COX-2 enzyme (2). This results in a pro-cytokine (inflammatory) cascade (2). In some hosts, this leads to Adult Respiratory Distress Syndrome (ARDS), which has a high lethality. The general time from symptom presentation to the subsequent development to ARDS is approximately 4–10 days with a progressive worsening of the condition during that time. It is not yet understood why some people when infected with COVID-19 get a relatively mild illness whereas others respond with an inflammatory response that can result in ARDS.

The COX-2 enzyme is not expressed in many tissues, including the lung, except when associated with inflammation. The role of the COX-2 enzyme has a poorly understood role in immunity (4). Viral infection COX-2 enzyme induction occurs in a complex process with the consequential immunity response being associated with the production of cytokines, inflammatory prostanoids, and increased vascular permeability (4).

AIMS OF TREATMENT

Any treatment using the bottom-up approach is aimed at improving the immunity of the entire host. Immunomodulation would then need to be protective, enhancing host immune health, and have treatment, anti-cytokine storm (anti-inflammatory) properties. It is likely that during bout of severe infection, different elements of the immune system will need enhancing, whereas other parts need suppressing. It is also likely that this may change during the infection, with unknown and unpredictable timeframes associated with this required alteration. Obviously, you need the "right" immunomodulating drug at the "right" time as administration at the wrong time could worsen clinical outcome. With current knowledge of any condition, and particularly a new emerging threat such as COVID-19, this is a difficult task.

In broad terms ARDS, the condition responsible for the mortality associated with COVID-19 may be classified into hyper- and hypo-inflammatory subphenotypes. With emerging virus infections these are generally considered to be hyper-inflammatory. Immediate cessation of smoking along with better hypertension/diabetes control would immediately assist in improving the bodies hyper-inflammation.

Other host responses that can be targeted with the specific need of trying to assist in decreasing ARDS presentations associated with the current COVID-19 pandemic include altering the lung endothelial cell membrane to decrease virus incorporation, decreasing the cytokine response with virus presentation to the endothelial cell membrane, altering the amount of lung host cell numbers to try and prevent virus incorporation, inhibiting the direct cell-to-cell spread (a common viral pathogenic mechanism), improving viral toxicity to prevent viral replication, and inhibiting inflammatory pathways after virus infection.

ENDOTHELIAL CELL MEMBRANE AND HMGCOA REDUCTASE INHIBITORS (STATINS)

It has been demonstrated that there is a 30% reduction in mortality on those patients admitting to hospital with pneumonia if they were taking HMGCoA reductase inhibitors "statins" prior to admission (5). With the study being performed retrospectively, it was also concluded that those patients taking both statins with a combination of either ACEI (Angiotensin Converting Enzyme Inhibitors) or an ARB (Angiotensin receptor blockers) experience even less lethality (6). Having both drugs in combination provided a more synergistic effect (6). Detailed information on the dose of statins was not disclosed, but there was slightly more protection from mortality when taking a higher dose of the statin simvastatin (5). The study was performed in the first decade of this century when simvastatin was more commonly prescribed than other statins, such as atorvastatin and rosuvastatin, and making a definitive conclusion about the effectiveness of one statin over another is thus not possible. Additionally, there was no statistical comparison performed between the different doses of statins.

In a randomized controlled trial (7) on patients diagnosed with ARDS there was no clinical benefit, morbidity or mortality, in those given Simvastatin compared to placebo. However, when the hyper-inflammatory sub phenotype of ARDS was separately analyzed there was improved survival with simvastatin compared to placebo, even when given late in the course of the disease (8). There have been more recent studies repeating this using the currently most commonly prescribed statins, Atorvastatin and rosuvastatin, with disappointing results in the use of Statins as a late treatment for ARDS (9).

Both statins and ACEI/ARB drugs are known to be anti-inflammatory and immunomodulatory. When the lung

endothelial cell barrier is breached by the virus this triggers a release of Angiopoietin (Angpt-2), which in turn increases the release of pro-inflammatory cytokines (1). Statins affect the Angpt/TIE2 axis and decrease Angpt-2 whereas the ARB's are direct angpt-2 antagonists (1). Statins have been demonstrated to inhibit airway inflammation, possibly by a pathway of attenuating RANTES release.

RANTES (regulated on activation, normal T-cell expressed and secreted) is now recognized to stimulate the influx of numerous inflammatory cells, including monocytes, eosinophils, and neutrophils. Additionally, statins attenuate viral dsRNA-induced AKT phosphorylation, which reduces viral replication.

THE USE OF STATINS

With respect to side-effect profile there is probably only limited downside in commencing statin drugs, considering the state of the current epidemic. However, if statins are to be used as a bottom-up approach, they need to be commenced with as much time prior to the viral infection as possible if this is to alter any underlying prevalent lung inflammation. The principal side-effects of commencing statins is associated with musculoskeletal side-effects, and these should be monitored in anyone commencing these drugs.

This recommendation for the use of Statins for treatment of SARS-CoV2 is difficult, as there are limited studies demonstrating clinical benefit once you are infected with the virus. The anti-inflammatory and immunomodulatory properties of statins need to be established prior to the host being infected.

ALTERATION IN THE AMOUNT OF HOST CELL NUMBERS TO INFLUENCE THE DISEASE

The ACE2 receptor on epithelial cells is the entry point for the SARS-CoV2 virus. The understanding of ACE2 has altered over recent years with it not being understood that overexpression results in better anti-hypertensive control. It is also known that ACE2 down regulates pro-inflammatory cytokines (2). In contrast, ACE2 deficiency will increase IL-6 and other similar pro-inflammatory proteins (10). There is a current argument about the role of ARB/ACEI drugs with COVID-19 that is beyond the scope of this analysis.

INHIBITION OF THE INFLAMMATORY PATHWAYS TO PREVENT ARDS

Viral infection activates a COX-2 inflammatory cascade that is most marked in the initial inflammatory phase (11).

Experiments demonstrated that administering a COX-2 inhibitor early in a disease course may enhance endogenous interferon, a protein that coordinates cellular anti-viral response (4). It was proposed that COX-2 inhibition could be an effective anti-viral therapy in humans to boost the anti-viral response provided it was given soon after the initiation of the infection (4). Mice with COX-2 enzyme deletion exhibited a reduced mortality to influenza (12).

With the respiratory distress associated with the H5N1 virus, the proinflammatory cascade was rapider and broader than those arising from other viral infections (13). As selective COX-2 inhibitors suppress hyper-induction of cytokines in the proinflammatory cascade, it was proposed that this knowledge could provide a basis for the possible development of novel therapeutic interventions for the treatment of hyper-inflammatory ARDS, as adjuncts to antiviral drugs (13).

Which COX-2 inhibitor should we trial for use? The rest of this analysis is devoted to that question.

PROPERTIES OF NSAIDs AND CORTICOSTEROIDS

All Non-Steroidal Anti-Inflammatory Drug (NSAIDs) exert their principal anti-inflammatory effect through their COX-2 inhibition. Of the commonly available NSAIDs, Diclofenac has as much COX-2 inhibition as Celecoxib and is more selective than Meloxicam (14). However, when the commonly prescribed doses are taken into account, Diclofenac 50 mg tds, with its shorter half-life (2 h), will result in more COX-2 inhibition when compared to the other COX-2 inhibitors like Refecoxib (highly selective Cox2 inhibitor) or Meloxicam (moderately selective Cox2 inhibitor) (15).

Commonly available NSAIDs that are selective COX-2 inhibitors have significantly longer half-lives (5–20 h) when compared to Diclofenac. Even Celecoxib, with the shortest half-life of 5 h of the selective COX-2 inhibitors, still needs a loading dose due its slow absorption with a plasma level peak at about day 3–4 (16). Diclofenac can reach therapeutic plasma levels far more rapidly due to its better absorption and shorter half-life. Additionally, acidic NSAIDs with high degree of protein binding (Diclofenac, Ibuprofen) more selectively accumulate and persist at sites of inflammation. This compares to the specific COX-2 inhibitors, which are non-acidic and are diffused homogenously throughout body. Diclofenac has been demonstrated to persist at the site of inflammation, as it is bound to COX-2, despite plasma clearance and kidney excretion (17).

When understanding inflammation, IL-10 (Interleukin-10) is immunosuppressive and downregulates IL-6 and the other proinflammatory cytokines. IL-6 mediates release of acute-phase proteins, which with SARS-CoV2 infection we consider as not desirable. In a randomized-controlled trial of post-surgical patients, those treated with Diclofenac, and compared to those not receiving a Diclofenac dosage, there was a decrease in IL-6, an increase in IL-10, and a smaller increase in CRP (18). It was observed that the IL-10 increased prior to the noted decrease in IL-6 and prior to the subsequent stress-induced cortisol action. The conclusion was that although prolonged high IL-10 has been associated with developing sepsis altering the immediate IL6-IL10 balance may be beneficial on reducing the acute inflammatory response (19). Other COX-2 inhibitors have been demonstrated to decrease IL-10, but these take a

longer period with upregulation occurring by day 6–7 (19). In the case of specific COX-2 inhibitors, this upregulation was considered a consequence of the normal homeostasis feedback loop rather than direct induction as proposed for the drug Diclofenac (20).

In conclusion, in looking for a COX 2 inhibitor to use as an anti-inflammatory agent in early viral disease, Diclofenac is probably the best due to its short half-life, rapid mechanism of action, and superior inhibition of COX-2 at therapeutic doses.

With the early response to the COVID-19 epidemic there has been some non-scientific reports of avoiding Ibuprofen—a less selective COX-2 inhibitor (15). It is known that taking COX-1 inhibitors can prime a subsequent inflammatory cascade, which is a property of most NSAIDs. Avoiding taking these medications prior to infection is therefore recommended. It is not known whether taking COX-2 inhibitors taken prior to infection would attenuate the COX-2 inhibition when it is required.

THE ANTI-INFLAMMATORY AND ANTI-VIRAL PROPERTIES OF DICLOFENAC MAKE IT SUITABLE FOR USE AS TREATMENT IN THE EARLY STAGE OF COVID-19 INFECTION

With infection there is a need for the endothelial cell endosomes to acidify in order to allow viral entry to the cell. Elevating endosomal pH by inhibiting cathepsin L, an important lysosomal endopeptidase enzyme, is the aim of another compound that has significant interest in the treatment of COVID-19 infections, hydroxychloroquine. NSAIDs, like Diclofenac, also inhibit Cathepsins L activity (21). In contrast, steroidal anti-inflammatories are not considered to inhibit this pathway (22).

It has been demonstrated that COX inhibition prevents Cytomegalovirus (CMV) from spreading the virus from cell to cell by a mechanism where the maturation and cell movement is blocked (23). To achieve this effect, high doses were needed (23). In this study another NSAID with a short half-life, Indomethacin, was used. Diclofenac, when compared to Indomethacin, also has a short half-life with similar COX-1-inhibition properties but superior COX-2-inhibition properties.

It had been considered that NSAID Cyclopentenone cyclooxygenase metabolites act against RNA viruses. COVID-19 is an RNA virus. To prove this, dogs known to have coronavirus infections were given Indomethacin and infected with SARS-CoV. When analyzed, there was a 1000-fold reduction in virus yield with the mechanism of effect being considered a blocking of viral RNA synthesis (24). However, this mechanism does not prime the host cell to raise a defense response against the virus but rather was useful when the virus had already entered the cell (24). Finally, the viral cytotoxic effect was seen at higher doses than would be needed for COX inhibition. Additionally, aspirin did not work in a similar manner (24).

Even though it is an old drug (1976) Diclofenac still has some unknown, somewhat novel, mechanisms of action which, when analyzed, may also assist in anti-viral (anti-COVID-19) action (25). Diclofenac blocks acid-sensing ion channels (25). The ion channel activity of SARS-CoV 3a protein is essential for activation of the pro-inflammatory NLRP3 inflammasome (26). Diclofenac also inhibits PPAR-γ (25). Notably, alveolar macrophages (AM) in the lungs constitutively express high levels of PPAR-γ to rapidly produce inflammatory cytokines following microbial challenge. The downregulation of PPAR-γ in the AM cells may result in beneficial functions under certain conditions (27), such as with the viral challenge to the lungs from SARS-CoV2. Diclofenac also inhibits Phospholipase-A2 (25) with binding by Diclofenac being demonstrated (28). Phospholipase-A2 is important in the pro-inflammatory response process. When comparing different drugs, Diclofenac reduced phospholipase-A2 activity by 93%, ketoprofen 90%, dexamethasone 62%, and methylprednisolone by 50% with weak inhibition of phospholipase-A2 activity being demonstrated by betamethasone and hydrocortisone (29). Finally, patients who died following SARS-CoV infection had elevated Phospholipase-A2 G2D (PLA2G2D) and were by the most part older (30). It is known that Phospholipase A2 increases in older people. Having these elevated PLA2G2D increases the level of immunosuppressive lipid mediators presumably to dampen the response to environmental antigens. It was considered that this adversely effected the protective innate response when this was required (30).

THE USE OF DICLOFENAC (OR OTHER COX-2 INHIBITORS) IN THE EARLY STAGE OF COVID-19 INFECTION

Diclofenac is inexpensive and available throughout the world, often without medical supervision. Although relatively safe, caution needs to be observed if using such a method of treatment on a population basis. Allergy to aspirin is a contra-indication with exacerbation of asthma and gastric mucosal lining damage being common side-effects. Caution needs to be exhibited in those people with impaired renal function. However, when compared to the current morbidity and mortality of the COVID-19 pandemic, amplified with the development of ARDS, and without effective other treatments at this stage it would be considered the downside risks could be managed.

CONCLUSION

This study used an analysis of the literature with a bottom-up approach to try and improve the host response to the looming (current) pandemic caused by the virus COVID-19. Following this analysis, we recommend the following: (1) optimize current health—cease (reduce) smoking, ensure adequate hypertension and diabetes control, and continue exercising; (2) with appropriate research being undertaken, commence an HMG CoA reductase inhibitor "statin" in a medium dose for its immunomodulatory and anti-inflammatory properties; and (3) consider using Diclofenac (or other COX-2

inhibition medications) for its anti-inflammatory and virus toxicity properties. For effectiveness and decreased risk, this needs to be in the early course of the disease (post infection and/or symptom presentation) and given in a high dose.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

REFERENCES

1. Fedson D. Treating the host response to emerging virus diseases: lessons learned from sepsis, pneumonia, influenza and Ebola. *Ann Transl Med*. (2016) 4:421. doi: 10.21037/atm.2016.11.03
2. Cheng V, Lau S, Woo P, Yuen K. Severe acute respiratory syndrome Coronavirus as an agent of emerging and reemerging infection. *Clin Micro Rev*. (2007) 20:660–94. doi: 10.1128/CMR.00023-07
3. Li W, Moore M, Vasilieva N. Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. *Nature*. (2003) 426:450–4. doi: 10.1038/nature02145
4. Kirkby N, Ziass A, Wright W, Jiao J, Chan MV, Warner TD, et al. Differential COX-2 induction by viral and bacterial PAMPs: consequences for cytokine and interferon responses and implications for anti-viral COX-2 directed therapies. *Biochem Biophys Res Comm*. (2013) 438:249–56. doi: 10.1016/j.bbrc.2013.07.006
5. Mortensen E, Nakashima B, Cornell E, Copeland LA, Pugh MJ, Anzueto A, et al. Population based study of statins, AT 2 receptor blockers (ARB's) and ACE inhibitors on pneumonia-related outcomes. *Clin Infect Dis*. (2012) 55:1466–73. doi: 10.1093/cid/cis733
6. Mortensen E, Anzueto A. Prior use of both a statin and ARB is associated with lower mortality for patients hospitalized with pneumonia. *Eur Respir J*. (2016) 48:OA33292016. doi: 10.1183/13993003.congress-2016.OA3329
7. McAuley DF, Laffey JG, O'Kane CM, Perkins GD, Mullan B, Trinder TJ, et al. Simvastatin in the acute respiratory distress syndrome. *N Engl J Med*. (2014) 371:1695–703. doi: 10.1056/NEJMoa1403285
8. Calfee CS, Delucchi KL, Sinha P, Matthay MA, Hackett J, Shankar-Hari M, et al. Acute respiratory distress syndrome subphenotypes and differential response to simvastatin: secondary analysis of a randomised controlled trial. *Lancet Respir Med*. (2018) 6:691–8.
9. Sinha P, Delucchi KL, Thompson BT, McAuley DF, Matthay MA, Calfee CS, et al. Latent class analysis of ARDS subphenotypes: a secondary analysis of the statins for acutely injured lungs from sepsis (SAILS) study. *Intensive Care Med*. (2018) 44:1859–69. doi: 10.1007/s00134-018-5378-3
10. Kazemi-Bajestani S, Patel V, Wang W, Oudit G. Targeting the ACE2 and apelin pathways are novel therapies for heart failure: opportunities and challenges. *Cardiol Res Prac*. (2012) 2012:823193. doi: 10.1155/2012/823193
11. Ricciotti E, FitzGerald GA. Prostaglandins and inflammation. *Arterioscler Thromb Vasc Biol*. (2011) 31:986–1000. doi: 10.1161/ATVBAHA.110.207449
12. Carey M, Bradbury J, Seubert R, Langenbach R, Zeldin DC, Germolec DR, et al. Contrasting effects of COX-1 and COX-2 deficiency on the host response to influenza A viral infection. *J Immunol*. (2005) 175:6878–84. doi: 10.4049/jimmunol.175.10.6878
13. Lee MY, Cheung CY, Peiris JS. Role of cyclooxygenase-2 in H5N1 viral pathogenesis and the potential use of its inhibitors. *Hong Kong Med J*. (2013) 19(Suppl. 4):29–35.
14. Altman R, Bosch B, Brune K, Patrignani P, Young C. Advances in NSAID development: evolution of diclofenac products using pharmaceutical technology. *Drugs*. (2015) 75:859–77. doi: 10.1007/s40265-015-0392-z
15. Van Hecken A, Schwartz J, Depre M, De Lepeleire I, Dallob A, Tanaka W, et al. Comparative inhibitory activity of refecoxib, meloxicam, diclofenac,ibuprofen and naproxen on COX-2 versus COX-1 in healthy volunteers. *J Clin Pharmacol*. (2000) 40:1109–20.
16. Brune K, Patrignani P. New insights into the use of currently available non-steroidal anti-inflammatory drugs. *J Pain Res*. (2015) 8:105–18. doi: 10.2147/JPR.S75160
17. Mahdy A, Galley H, Abdel-Wahed A, el-Korny KF, Sheta SA, Webster NR. Differential modulation of interleukin-6 and interleukin-10 by diclofenac in patients undergoing major surgery. *Br J Anae*. (2002) 88:797–802. doi: 10.1093/bja/88.6.797
18. Stolina M, Sharma S, Lin Y, Dohadwala M, Gardner B, Luo J, et al. specific inhibition of cyclooxygenase 2 restores antitumor reactivity by altering the balance of IL-10 and IL-12 synthesis. *J Immunol*. (2000) 164:361–70. doi: 10.4049/jimmunol.164.1.361
19. Sriramula A, Xia H, Xu P, Lazartigues E. Brain-targeted ACE2 overexpression attenuates neurogenic hypertension by inhibiting COX mediated inflammation. *Hypertension*. (2015) 65:577–86. doi: 10.1161/HYPERTENSIONAHA.114.04691
20. Blanco F, Guitian R, Moreno J, de Toro FJ, Galdo F. Effect of anti-inflammatory drugs on the COX-1 and COX-2 activity in human articular chondrocytes. *J Rhuematol*. (1999) 26:1366–73.
21. Raghav N, Kamboj RC, Singh H. Effect of some steroidal & non-steroidal anti-inflammatory drugs on purified goat brain cathepsin L. *Indian J Med Res*. (1993) 98:188–92.
22. Zielińska K, Van Moortel L, Opdenakker G, De Bosscher K, Van den Steen PE. Endothelial response to glucocorticoids in inflammatory diseases. *Front Immunol*. (2016) 7:592. doi: 10.3389/fimmu.2016.00592
23. Schroer J, Shenk T. Inhibition of cyclooxygenase activity blocks cell-to-cell spread of human cytomegalovirus. *Proc Natl Acad Sci USA*. (2008) 105:19468–73. doi: 10.1073/pnas.0810740105
24. Amici C, Di Caro A, Ciucci A, Chiappa L, Castilletti C, Martella V, et al. Indomethacin has a potent antiviral activity against SARS Coronavirus. *Antivir Ther*. (2006) 22:1021–30.
25. Gan TJ. Diclofenac: an update on its mechanism of action and safety profile. *Curr Med Res Opin*. (2010) 26:1715–31. doi: 10.1185/03007995.2010.486301
26. Chen I, Moriyama M, Chang M, Ichinohe T. Severe acute respiratory syndrome Coronavirus viroporin 3a activates the NLRP3 inflammasome. *Front Microbiol*. (2019) 10:50. doi: 10.3389/fmicb.2019.00050
27. Huang S, Zhu B, Cheon I, Goplen NP, Jiang L, Zhang R, et al. PPAR-γ in macrophages limits pulmonary inflammation and promotes host recovery following respiratory viral infection. *J Virol*. (2019) 93:e00030-19. doi: 10.1128/JVI.00030-19
28. Singh N, Jabeen T, Sharma S, Somvanshi RK, Dey S, Srinivasan A, et al. Specific binding of non-steroidal anti-inflammatory drugs (NSAIDs) to phospholipase A2: structure of the complex formed between phospholipase A2 and diclofenac at 2.7 A resolution. *Acta Crystallogr D Biol Crystallogr*. (2006) 62(Pt 4):410–6. doi: 10.2210/pdb2b17/pdb
29. Mäkelä A, Kuusi T, Schröder T. Inhibition of serum phospholipase-A2 in acute pancreatitis by pharmacological agents in vitro. *Scand J Clin Lab Invest*. (1997) 57:401–7. doi: 10.3109/00365519709084587
30. Vijay R, Hua X, Meyerholz D, Miki Y, Yamamoto K, Gelb M, et al. Critical role of phospholipase group IID in age-related susceptibility to severe acute respiratory syndrome – CoV infection. *J.Exp.Med*. (2015) 212:1851–68. doi: 10.1084/jem.20150632

Conflict of Interest: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Medicine

ORIGINAL RESEARCH
published: 03 September 2020
doi: 10.3389/fmed.2020.556366

What Can We Estimate From Fatality and Infectious Case Data Using the Susceptible-Infected-Removed (SIR) Model? A Case Study of Covid-19 Pandemic

Semra Ahmetolan[1], Ayse Humeyra Bilge[2], Ali Demirci[1], Ayse Peker-Dobie[1,2] and Onder Ergonul[3]*

[1] Department of Mathematics, Faculty of Science and Letters, Istanbul Technical University, Istanbul, Turkey, [2] Department of Industrial Engineering, Faculty of Engineering and Natural Sciences, Kadir Has University, Istanbul, Turkey, [3] Infectious Diseases and Clinical Microbiology Department, School of Medicine, Koc University, Istanbul, Turkey

OPEN ACCESS

Edited by:
Abdallah Samy,
University of Kansas, United States

Reviewed by:
Ali Dinler,
Istanbul Medeniyet University, Turkey
Hasan Guclu,
Istanbul Medeniyet University, Turkey

***Correspondence:**
Ali Demirci
demircial@itu.edu.tr

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: *27 April 2020*
Accepted: *12 August 2020*
Published: *03 September 2020*

Citation:
Ahmetolan S, Bilge AH, Demirci A, Peker-Dobie A and Ergonul O (2020) What Can We Estimate From Fatality and Infectious Case Data Using the Susceptible-Infected-Removed (SIR) Model? A Case Study of Covid-19 Pandemic. Front. Med. 7:556366. doi: 10.3389/fmed.2020.556366

The rapidly spreading Covid-19 that affected almost all countries, was first reported at the end of 2019. As a consequence of its highly infectious nature, countries all over the world have imposed extremely strict measures to control its spread. Since the earliest stages of this major pandemic, academics have done a huge amount of research in order to understand the disease, develop medication, vaccines and tests, and model its spread. Among these studies, a great deal of effort has been invested in the estimation of epidemic parameters in the early stage, for the countries affected by Covid-19, hence to predict the course of the epidemic but the variability of the controls over the course of the epidemic complicated the modeling processes. In this article, the determination of the basic reproduction number, the mean duration of the infectious period, the estimation of the timing of the peak of the epidemic wave is discussed using early phase data. Daily case reports and daily fatalities for China, South Korea, France, Germany, Italy, Spain, Iran, Turkey, the United Kingdom and the United States over the period January 22, 2020–April 18, 2020 are evaluated using the Susceptible-Infected-Removed (SIR) model. For each country, the SIR models fitting cumulative infective case data within 5% error are analyzed. It is observed that the basic reproduction number and the mean duration of the infectious period can be estimated only in cases where the spread of the epidemic is over (for China and South Korea in the present case). Nevertheless, it is shown that the timing of the maximum and timings of the inflection points of the proportion of infected individuals can be robustly estimated from the normalized data. The validation of the estimates by comparing the predictions with actual data has shown that the predictions were realized for all countries except USA, as long as lock-down measures were retained.

Keywords: COVID-19, SIR model, parameter estimation, mathematical models, epidemiology

INTRODUCTION

The Coronavirus disease 2019 (Covid-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a highly contagious disease affecting huge numbers of people all over the world. The earliest case was identified in China in December 2019. After the first diagnosis, the disease has spread very quickly to other countries, in spite of efforts to slow and stop the transmission of COVID-19, such as self-isolation, quarantine, social distancing, contact tracing, and travel limitations. As a result of its rapid spread and very high infection rates, the World Health Organization (WHO) declared Covid-19 a pandemic in March 2020 (1).

As of April 2020, even though the pandemic has passed its early stage and there are 90% fewer cases in China as a consequence of successful containment measures, the disease is rapidly expanding in Europe, America, Asia, Middle East, and Africa. Despite the application of travel restrictions by many countries, there have been no substantial delays in the arrival of the pandemic in non-affected areas, as in the case of the H1N1 epidemic in 2009 (2).

A great deal of effort has been invested in the estimation of epidemic parameters of Covid-19 in the early stage for China and some other countries (3–13). In (3), the authors analyzed the temporal dynamics of the disease in China, Italy and France in the period between 22nd of January and 15th of March 2020. In (4), the potential for sustained human-to-human transmission to occur in locations outside Wuhan is assessed based on the estimations of how transmission in Wuhan varied between December, 2019, and February, 2020. The difficulties related to the accurate predictions of the pandemic is discussed in (5). In (6), the authors used phenomenological models that were developed for previous outbreaks to generate and assess short-term forecasts of the cumulative number of confirmed reported cases in Hubei province and for the overall trajectory in China (7). Epidemic analysis of the disease in Italy is presented in (8) by means of dynamical modeling (9). Forecasting Covid-19 is investigated in (10) by using a simple iteration method that needs only the daily values of confirmed cases as input. A cumulative distribution function (CDF) and its first derivative are used to predict how the pandemic will evolve in (11). In (12), the authors proposed a segment Poisson model for the estimation. In (13), a meta-population model of disease transmission in England and Wales was adapted to predict the timing of the peak of the epidemic. In addition, it was shown that the change in the epidemic behavior of various countries can be traced by the use of data driven systems (14).

One of the common features of these works is the existence of variations in these parameter estimations. In the present work, the determination of the following parameters is discussed:

1) The Basic Reproduction Number $\Re_0$,
2) The mean duration of the infectious period T,
3) The time t_m (days) at which the number of infectious cases reaches its maximum, i.e., the first derivative of I(t) is zero,
4) The time t_a (days) at which the rate of increase in the number of infectious cases reaches its maximum, i.e., the time at which the second derivative of I(t) is zero and the first derivative is positive,
5) The time t_b (days) at which the rate of decrease in the number of infectious cases reaches its maximum, i.e., the time at which the second derivative of I(t) is zero and the first derivative is negative.

By employing the Susceptible-Infected-Removed (SIR) model, we show that the quantity that can be most robustly estimated from normalized data, is the timing of the maximum and timings of the inflection points of the proportion of infected individuals. These values correspond to the peak of the epidemic and to the highest rates of increase and the highest rates of decrease in the number of infected individuals. The stability of the estimations is discussed by comparing predictions based on data with long time spans.

DATA AND METHODS

Publicly accessible data that have been released by the state offices of each country are used for the analysis. The data set of each country is collected according to published official reports and available at the website http://www.worldometers.info/coronavirus/ (access: April 27, 2020). Updated data are also available at the website http://epikhas.khas.edu.tr/. Data used for the analysis covers the period January 22-April 18, 2020 and in the following, "Day 1" corresponds to January 22, 2020. The analysis uses Susceptible-Infected-Removed (SIR) model (15) and solutions are obtained by numerical methods. Updated data covering the period 19 April-1 July is used to assess the performance of the model.

SIR Model

The Susceptible-Infected-Removed (SIR) model is a system of ordinary differential equations modeling the spread of epidemics in a closed population, under the assumption of permanent immunity and homogeneous mixing (15). These equations are

$$S' = -\beta\, S\, I, \quad I' = \beta\, S\, I - \eta\, I, \quad R' = \eta\, I \tag{1}$$

Since the right hand sides of these equations add up to zero, the sum S + I + R is a constant that is equal to the total number of individuals in the population. Thus by normalizing, we may assume that S, I, and R are proportions of individuals in respective groups. Since the Covid-19 infection has an incubation period, the right model to use is the SEIR system. But, in previous work (16) it was shown that the parameters of the SEIR model cannot be determined from the time evolution of the normalized curve of removed individuals. Thus, the SEIR model should not be used in the absence of additional information that might be obtained by clinical studies. In the present work, since we assume no clinical information we will use the SIR model, with necessary modifications for the interpretation of the results, as indicated in (16).

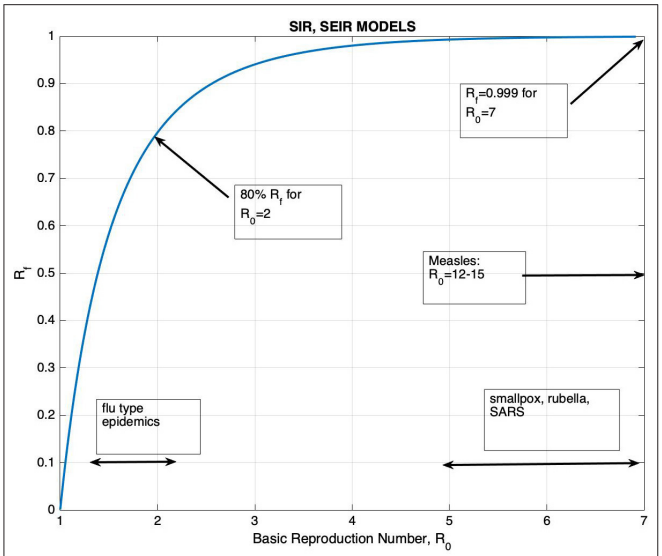


FIGURE 1 | The graph of R_f, the final proportion of individuals that would be affected by the disease, vs. the Basic Reproduction Number $\Re_0$.

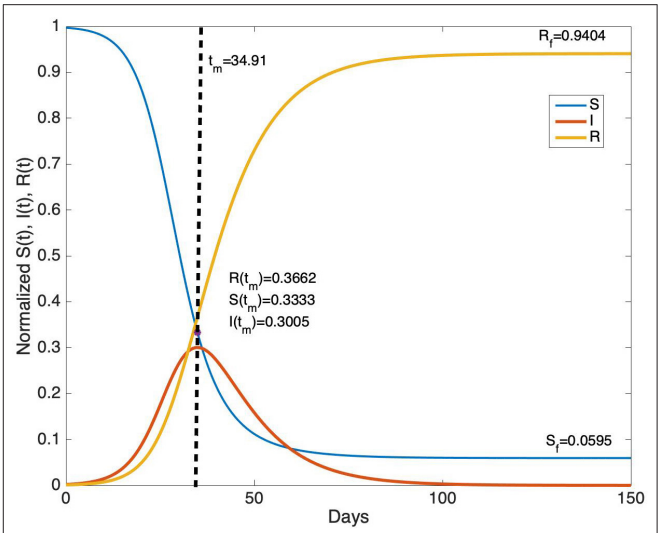


FIGURE 2 | The time evolution of S(t), I(t), and R(t) for $\Re_0 = 3$, T = 10 days and $R(0) = 10^{-3}$.

Relation Between the Basic Reproduction Number and the Total Number of Removed Individuals

The ratio β/η, called the Basic Reproduction Number and denoted as $\Re_0$, is the key parameter in both the SIR and SEIR models. This number is related to the growth rate of the number of infected individuals in a fully susceptible population and determines the final value of R denoted by R_f that is the proportion of individuals that will be affected by the disease. This proportion includes individuals who gain immunity without showing symptoms, those who are treated, as well as disease-related fatalities. The reciprocal of the parameter η, $T = 1/\eta$ is considered as a representative of the mean infectious period.

The relation between $\Re_0$ and R_f is determined as follows. Note that R(t) is a monotonically increasing function, and hence it can be used as an independent variable, instead of t. The derivative of S with respect to R is given by

$$dS/dR = S'/R' = -\beta/\eta\, S = -\Re_0\, S. \qquad (2)$$

Assuming initial conditions $S \to 1$ and $R \to 0$ as t approaches negative infinity, on can integrate and obtain $S(t) = e^{-\Re_0 R(t)}$. Then, as t approaches positive infinity, since $I \to 0$, $S + R = 1$ yields

$$R_f + e^{-\Re_0 R_f} = 1. \qquad (3)$$

$\Re_0$ can be solved from this equation as a function of R_f, and their relation is displayed on **Figure 1**.

The graph of R_f vs. $\Re_0$ is shown on **Figure 1**, together with the ranges of $\Re_0$ for well-known diseases. It can be seen that for $\Re_0 > 2.5$, R_f is >90%. The figure also shows that the increase in R_f with respect to $\Re_0$ is very slow for $\Re_0 > 3$. It is generally accepted that the $\Re_0$ for Covid-19 is >3 despite all containment measures (17–19). Thus, unless vaccination is applied, one would expect that at least 95% of the population would be affected by the disease. In addition, the knowledge of its precise value would have little effect on the planning of healthcare measures. It should also be kept in mind that containment measures provide a temporary control of the spread of the epidemic, just to the point of reducing the burden of the epidemic to a manageable size.

According to the Centers for Disease Control and Prevention (CDC), at the time we completed the data collection phase of our research, it was still unknown when viral shedding begins or how long it lasts for, and nor is the period of COVID-19's infectiousness known. Like infections with MERS-CoV and SARS-CoV, SARS-CoV-2 RNA may be detectable in the upper or lower respiratory tract for weeks after illness onset, though the presence of viral RNA is no guarantee of the presence of the infectious virus. It has been reported that the virus was found without any symptoms being shown (asymptomatic infections) or before symptoms developed (pre-symptomatic infections) with SARS-Cov-2, though the role they may play in transmission remains unknown. According to prior studies, the incubation period of SARS-CoV-2, like other coronaviruses, may last for 2–14 days[1].

To illustrate an example for an SIR model, $\Re_0$, T, and R(0) are chosen as 3, 10, and 10^{-3}, respectively and the related graphs are given on **Figure 2**.

From **Figure 2**, it can be seen that for parameter values $\Re_0 = 3$, T = 10 day, the duration of the epidemic is about 100 days. The peak of the epidemic occurs approximately at day 35. Note that the derivative of I(t) vanishes at time t_m when $S(t_m) = 1/\Re_0$. In this example, $S(t_m) = 0.3333$, $I(t_m) = 0.3005$, and $R(t_m) = 0.3662$.

[1]https://www.cdc.gov/coronavirus/2019-ncov/hcp/faq.html (accessed April 27, 2020).

The final values of S(t) and R(t) are $S_f = 0.0595$ and $R_f = 0.9404$ at the end of the epidemic.

Representative Data for the Proportion of Removed Individuals

It is in general accepted that the number of fatalities represents the number of removed individuals and the number of confirmed cases represents the number of infected individuals. In the initial phase of the epidemic, little information was available on the proportionality constants, but as long as they don't change in time, one can work with the normalized case reports and normalized fatalities and look for the determination of the epidemic parameters from the shape of these normalized curves. In section Estimation of the SIR Model Parameters, it will be shown that for the Covid-19 data, total cases would be a better representative of the number of removed individuals.

OVERVIEW OF DATA

According to the SIR model, given by the equations in (1), the rate of change of the number of removed individuals is proportional to the number of infectious cases. In terms of observations, this corresponds to the fact that the ratio of, for example, daily fatalities to daily infectious cases should be constant. In the literature on the analysis of historical epidemics, fatality reports are usually the only available data, hence models are necessarily based on the assumption that cumulative fatalities represent cumulative number of removed individuals. For the Covid-19 pandemic, as daily fatality and infectious case reports are available, further evaluation of the representation of R(t) in terms of fatality data is presented. Daily infections and total fatalities are displayed on **Figure 3**, for all countries.

Time Evolution of Daily Infections and Total Fatalities

Normalized daily infectious cases and total fatalities are shown on **Figure 3**.

From **Figure 3**, it can be seen that the epidemic cycle has been completed in China over the course of about 70 days. The jump in total fatalities is due to a change in the reporting scheme. As our analysis is based on total infectious cases, this change has no effect on the models. For South Korea, the epidemic is in a state of slow decrease at the end of about 60 days, but the rate of infections is still high. This qualitative behavior is an indication of the fact that $\Re_0$ for South Korea is expected to be much higher than the one for China (7, 20). For France, Germany and Iran, the epidemic is in the decline phase. For the rest of the countries, further analysis is needed in order to assess epidemic phase.

ESTIMATION OF THE SIR MODEL PARAMETERS

As noted above, the knowledge of $\Re_0$ determines the total proportion of individuals that would be affected, R_f.

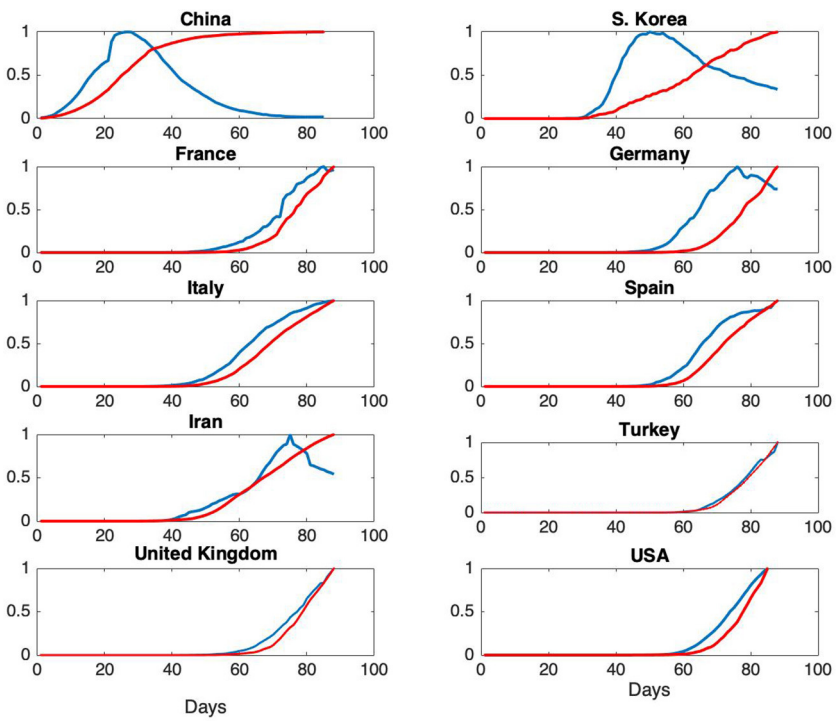


FIGURE 3 | Normalized daily case reports (Blue) and normalized total fatalities (Red) for each country. The horizontal axis represents days starting from January 22 and vertical axis represents ratios.

Furthermore, the peak of I(t) occurs at the time t_m, at which the proportion of susceptible individual falls to the value $1/\Re_0$. This information is useful for the determination of the proportion of people that have to be vaccinated in order to drag the proportion of susceptible individuals below this threshold. The Basic Reproduction Number is "defined" as the number of new infections per unit time in a fully susceptible population. Thus, it is a quantity that might be measured by direct on-site observations. On the other hand, the knowledge of $\Re_0$ by itself does not give any information on the timing of the progress of the epidemic.

It will be seen that $\Re_0$ and T can be estimated only for China where the spread of the epidemic is over. For other countries, $\Re_0$ and T cannot be estimated from the normalized data, but the timings of the key events, t_m, t_a, and t_b can be determined quite reliably.

Methods for Estimating the Parameters $\Re_0$, T, t_m, t_a, and t_b

These parameters are determined by a "brute force" approach: The models are run for a broad range of parameters. Then the difference between data and the model is compared by using various norms. Finally, the models that match data within 5% are selected. If the scatter plot of the errors vs. the parameter to be estimated has a sharp minimum, it is concluded that the corresponding parameter can be determined from the shape of the normalized data.

The parameter ranges for the SIR model are

$$1.5 < \Re_0 < 10, \quad 2 < T < 30, \tag{4}$$

and the initial values are chosen as

$$R_{ini} = 10^{-k}, \quad S_{ini} = e^{-(\beta/\eta)R_{ini}}, \quad I_{ini} = 1 - S_{ini} - R_{ini}, \tag{5}$$

where $1 < k < 10$. For South Korea, these parameter ranges are extended appropriately.

Selection of Representative Data for R(t)

In the SIR model, since $R' = \eta I$; that is, the rate of change in the number of removed individuals is proportional to the number of infected individuals, it is expected that the cumulative cases are proportional to cumulative fatalities. Thus, the SIR model predicts the simultaneity of the daily fatalities and daily infections. The verification of this fact requires the availability of data both for infections and for fatalities. The data for the 2009 H1N1 epidemic collected at certain major hospitals (21) is valuable in the sense of reflecting information on both infections and fatalities. The peculiarity of this data is a shift of about 8 days between total infections and total fatalities, the peak of infections occurring 8 days prior to the peak of fatalities. This time shift was explained by a multi-stage SIR model (22).

Cumulative cases and cumulative fatalities for Covid-19 do not show such a clear time shift. On the contrary, in China and Korea, fatalities increase faster than infections. In Germany, there is a slight lead for infections, while for other countries the two curves more or less coincide. The lead of fatalities over infections that is observed in China and in Korea is an unexpected fact, which is possibly due to the irregularities in the statistics, in medical treatment practices, etc. We should also note that the progression of the Covid-19 epidemic is unique in the sense that new treatment methods are applied during the initial phase in China and these methods have been applied in other countries.

For China, several programs were run, first by fitting the predicted R(t) to the total fatality data, then to the cumulative infectious case data. In the first case, about 700 models fitting cumulative fatalities within 5% error and about 3,000 models that fit cumulative infections within 5% error are found. Furthermore, in the latter case, the minima for the quantities that were aimed to be determined were much sharper. For South Korea, as it will be explained later, the model matching was not successful. For other countries, as the difference between total infections and total fatalities was negligible, total infections are used as a representative of R(t) of the SIR model.

Our main result is that it is not possible to determine the Basic Reproduction Number and the mean duration of the infectious period from the shape of the *normalized* data (unless there are reasonable estimates for either of these parameters). In order to make a reliable determination of the parameters $\Re_0$ and T by using the early stage data, a certain period of time has to pass. This period is ∼70 days for 2009 A(H1N1) epidemic (22). However, this period for Covid-19 is still uncertain. This is possibly the reason why the parameters for countries other than China and South Korea can not be established. On the other hand, the timings of the peak of the infectious cases, the peak of the rate of increase and the rate of decrease of the infectious cases can be determined more precisely from the shape of the normalized data.

Simulations for SIR Models With $\Re_0/T$ = Constant

The 'best' estimations of the parameters $\Re_0$ and T lie on a curve that is nearly linear when a SIR model is used to fit the data of an epidemic. This fact has been observed in previous work (23), in the study of the H1N1 epidemic and it was explained by the fact that the duration of the epidemic pulse (appropriately defined in terms of a fraction of the peak of infections) was nearly invariant for values of $\Re_0$ and T, with $\Re_0/T$ constant.

In order to visualize this situation, the solutions of this system of differential equations of the SIR model (1) for parameter range $3 < \Re_0 < 20$, and $\beta = \Re_0/T = 1/5$ are obtained. The graphs of normalized solutions (after an appropriate time shift) are given in **Figure 4**.

RESULTS FOR EACH COUNTRY

The scatter plots of the mean infectious period T vs. $\Re_0$, and the scatter plots of the modeling error vs. the parameters are presented in **Figures 5–9** where I_t and I_{tt} represent the values of the first and the second derivatives of I(t) at the last day of the data April 18, 2020, respectively. The error stands for the relative error between the normalized R(t) of the model and normalized total infectious cases, in the L_2 norm.

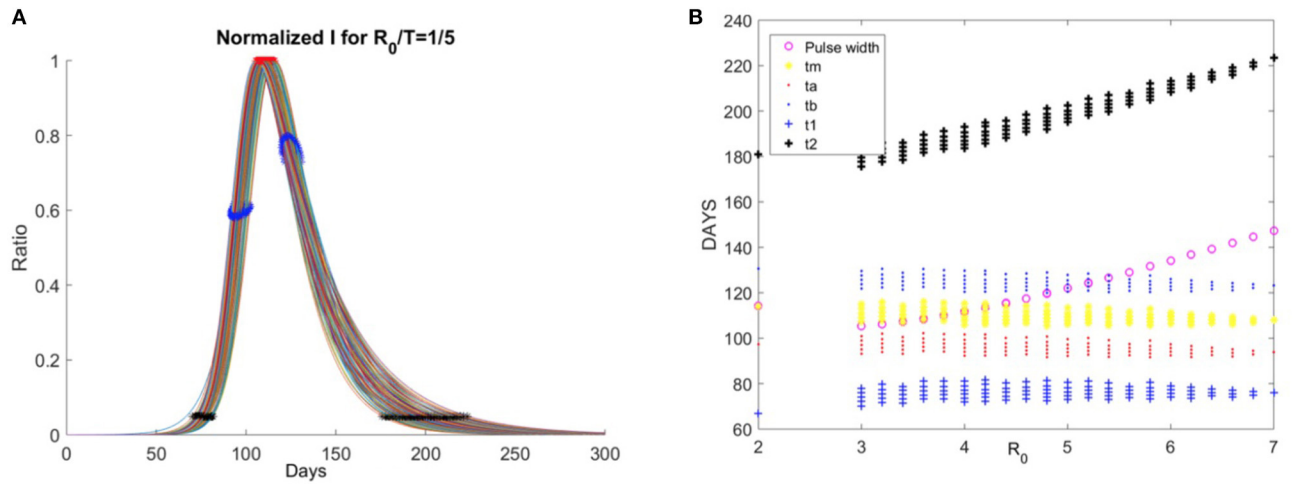


FIGURE 4 | (A) Normalized values of I(t) for $3 < \Re_0 < 20$, and $\beta = \Re_0/T = 1/5$, together with the inflection points (t_a, t_b), the peak point (t_m), and the timing of the initial (t_1) and final (t_2) points when the 5% of maximum value I(t) epidemic, **(B)** dependency of t_1, t_a, t_m, t_b, t_2 on $\Re_0$.

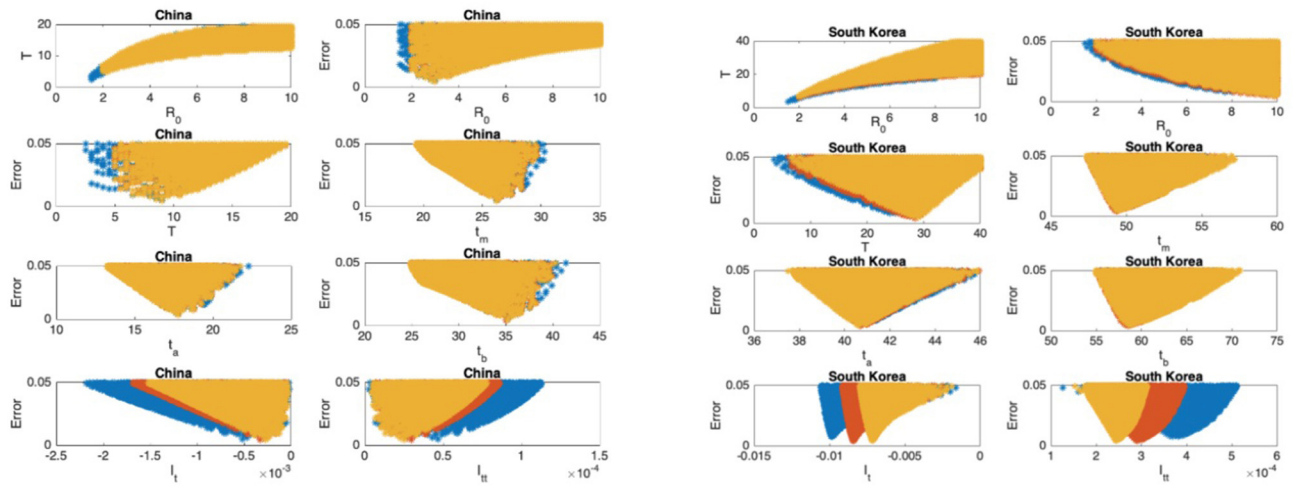


FIGURE 5 | China: the 7th and 8th graphs indicate that the epidemic is in Phase IV. South Korea: the 7th and 8th graphs indicate that the epidemic is in Phase IV. The values for $\Re_0$ and T don't seem to fall in reasonable ranges and the data for South Korea should be studied more closely.

Scatter Plot of the Mean Duration of the Infectious Period vs. the Basic Reproduction Number

In **Figures 5–9**, the first graph, in the upper left of the panel is the scatter plot of the mean duration of the infectious period, T, with respect to the basic reproduction number $\Re_0$, for models that fit data within 5% error in the norm described above. For all countries, the "best" parameters lie on a curve, instead of being agglomerated around a mean. This indicates that although the SIR model fitting normalized data is unique, the parameters $\Re_0$ and T cannot be determined precisely from normalized data. The colors blue, red, and yellow in **Figures 5–9** represent the results according to whether the last day of the analysis, t_f, is 78, 83, and 88, respectively.

Scatter Plot of the Modeling Error vs. the Basic Reproduction Number and vs. the Mean Duration of the Infectious Period

In **Figures 5–9**, the second (first row, right panel) and the third (second row, left panel) graphs display the scatter plot of the modeling error with respect to $\Re_0$ and T, respectively. For China, there are well-defined minima in the modeling errors at nearly $\Re_0 = 3$ and $T = 9$. For South Korea, the minima of the error in $\Re_0$ seems to be located beyond $\Re_0 = 8$, and the minimal error in T corresponds to $T = 25$ approximately. These parameter values are not in the ranges reported in the literature. Data for South Korea shows different characteristics, that might be due to the strategy of extensive testing and filiation, as opposed to lock-down measures. An indication of

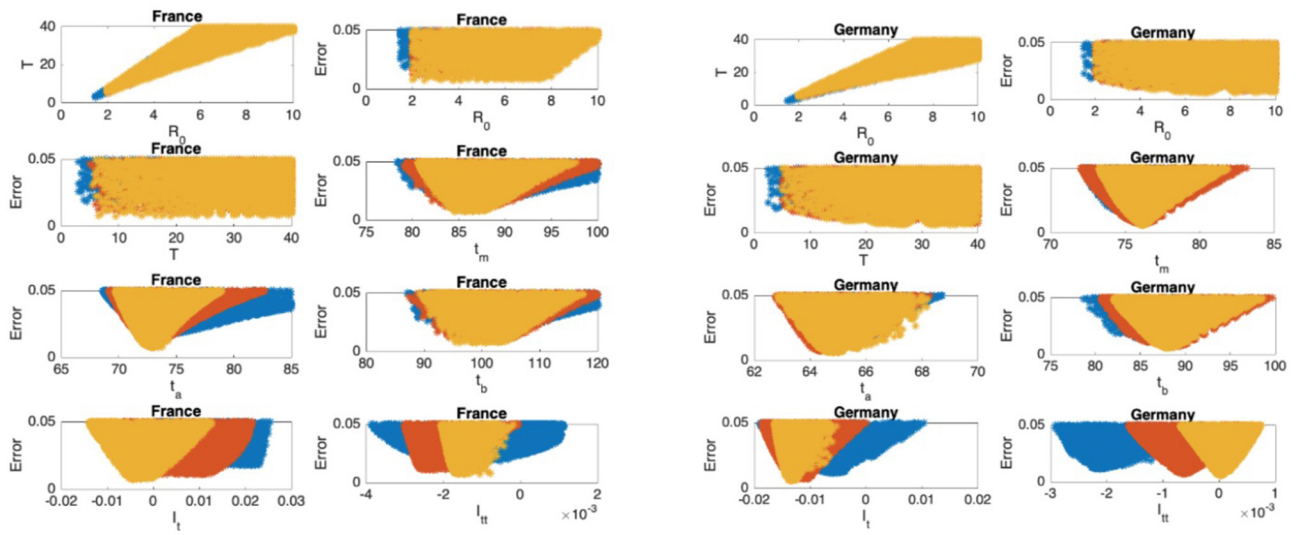


FIGURE 6 | France: the 7th and 8th graphs indicate that the epidemic is at the beginning of Phase III. Germany: the 7th and 8th graphs indicate that the epidemic is at the beginning of Phase IV.

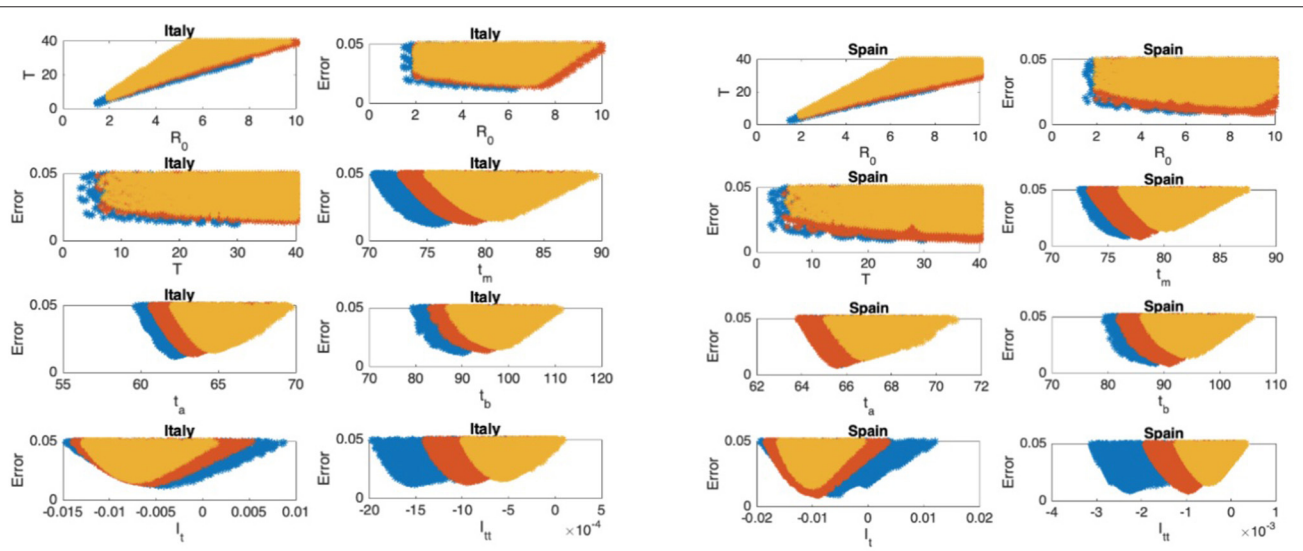


FIGURE 7 | Italy: the 7th and 8th graphs indicate that the epidemic is in Phase III. Spain: the 7th and 8th graphs indicate that the epidemic is in Phase III.

extensive testing policy is the fact that ∼27.4 percent of confirmed coronavirus patients in South Korea were in their 20s, showing that asymptomatic cases are also included in the statistics. For all of the remaining countries, the ranges of $\Re_0$ and T corresponding minimal modeling errors are too large to attempt any reasonable estimation for these parameters. If either $\Re_0$ or T is estimated by using alternative methods (medical observations etc.), it would be possible to obtain better estimates and improve the model by bootstrapping.

Timing of the Peak of the Maximum for I(t)

The fourth (second row, right panel) graph in **Figures 5–9** shows the scatter plot of the modeling error vs. t_m, the timing of the peak of the number of infections. For all of the countries analyzed, this parameter can be estimated quite sharply. In order to study the reliability of this estimation, the model matching process is repeated for $t_f = 78$, 83, and 88.

Timing of the Inflection Points of I(t)

The ratio of infected individuals I(t) has two inflection points. The first inflection point (t_a) is located at the left of the maximum (t_m) whereas the second one (t_b) is located at the right of t_m. t_a and t_b correspond to the highest rate of increase and decease in I(t), respectively. In **Figures 5–9**, the right and left panels of the third row display scatter plot of the error in these quantities. Their variation with respect to t_f is also investigated.

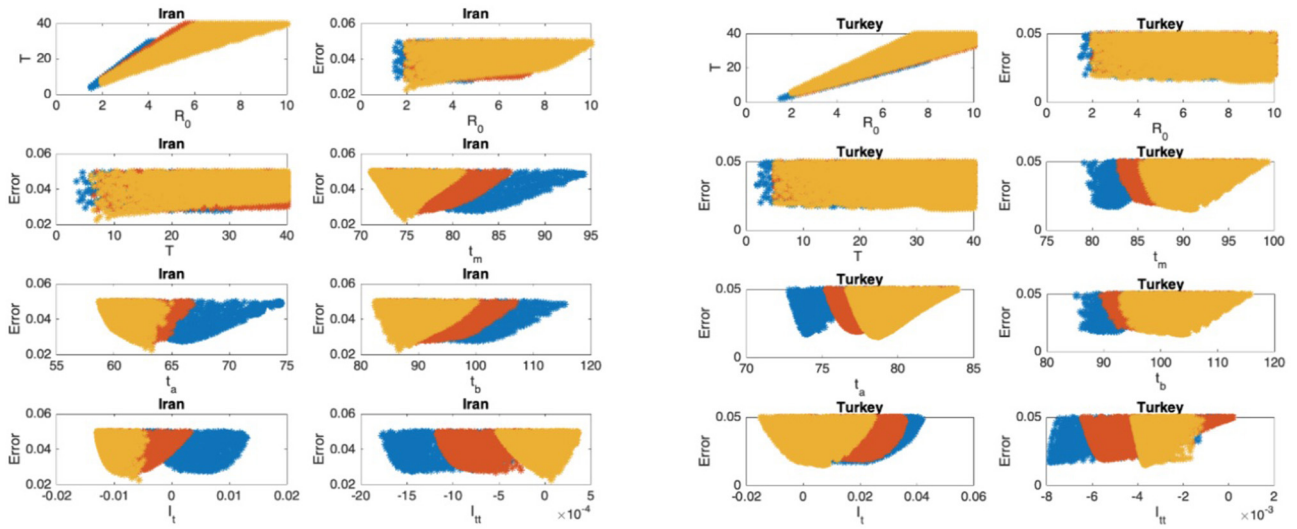


FIGURE 8 | Iran: the 7th and 8th graphs indicate that the epidemic is at the beginning of Phase IV. Turkey: the 7th and 8th graphs indicate that the epidemic is at the end of Phase II.

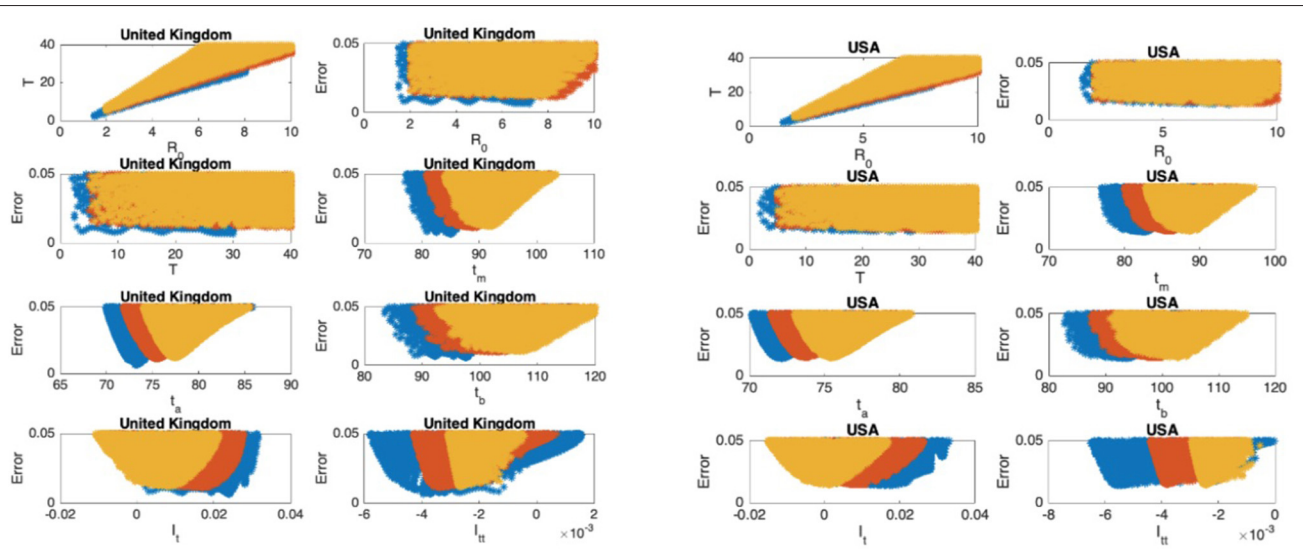


FIGURE 9 | United Kingdom: the 7th and 8th graphs indicate that the epidemic is in Phase II. United States: the 7th and 8th graphs indicate that the epidemic is at the end of Phase II.

Final Values of the First and Second Derivatives of I(t)

The values of the first and second derivatives at t_f are shown on the fourth row, left and right panels, respectively. If the first derivative is positive (negative), the I(t) is in the rising (falling) phase, while if the second derivative is positive (negative) the curve is concave up (down).

The epidemic phases which are shown in **Figure 10**, are categorized by the sign of the first and the second derivatives of I(t) as follows

1. Phase I: slow increase $\left(\frac{dI}{dt} > 0, \frac{d^2I}{dt^2} > 0\right)$
2. Phase II: fast increase $\left(\frac{dI}{dt} > 0, \frac{d^2I}{dt^2} < 0\right)$
3. Phase III: fast decrease $\left(\frac{dI}{dt} < 0, \frac{d^2I}{dt^2} > 0\right)$
4. Phase IV: slow decrease $\left(\frac{dI}{dt} < 0, \frac{d^2I}{dt^2} > 0\right)$

Estimation of parameters for each country and for $t_f = 88$ is summarized in **Table 1**.

MODELING VS. FORECAST

In section Results for Each Country, it can be seen that although $\Re_0$ and T cannot be determined, it was possible to estimate t_m,

t_a, and t_b quite sharply from data. In this section, the reliability of these estimates is discussed by comparing predictions based on data with different time spans.

The best SIR models fitting data for 78, 83, and 88 days are obtained, and data and graphs of 10 best models for each time span are plotted in **Figures 11**, **12**. For China and South Korea, for which the epidemic cycle is more or less complete, estimations based on time spans varying by 5 days give the same result as can be observed in **Figure 11**.

On the other hand, for those countries that are as yet before or around the peak of the epidemic, the situation may be different, as can be observed in **Figure 12**.

Accuracy of estimates was ascertained through comparison with the real data between 19th April and 1st July. These comparisons are given in **Figures 11**, **12** as red dashed curves. The observations are as follows.

When the initial analysis was performed, China, and South Korea were in Phase 4. Our estimates and the real data for both countries are consistent.

The estimate for France is not consistent with the real data post day 88. French authorities loosened quarantine restrictions on 11th May (day 111). This event may be the reason for the fluctuations in the number of infectious cases.

The estimates for Germany and Iran are consistent with the real data. However, Germany is going through the third and the fourth phases faster than expected. Besides, the active infectious cases post 3rd May (day 103) show a continuous increase. The decrease in the active infectious cases up to this date was close to our estimates.

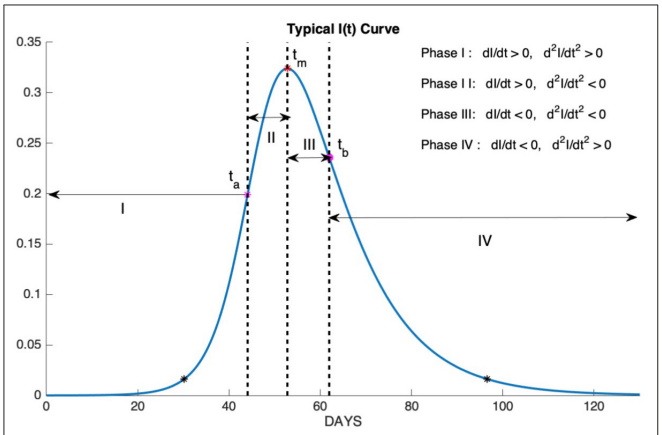


FIGURE 10 | Phase I: slow increase, Phase II: fast increase, Phase III: fast decrease, Phase IV: slow decrease.

TABLE 1 | Timing of the phases of the epidemic.

	China ($t_f = 85$)	South Korea	France	Germany	Italy	Spain	Iran	Turkey	United Kingdom	United States
$\Re_0$	3	8	–	–	–	–	–	–	–	–
T	9	25	–	–	–	–	–	–	–	–
$t_{m\ (Estimated)}$	26	50	86	76	81	81	75	88–92	87–92	90–92
$t_{m\ (Real)}$	27	50	84	76	89	93	75	93	N/A	Not occur
t_a	18	41	74	65	65	67	63	78	77	75
t_b	35	59	95–104	88–90	97–100	93–95	86–88	96–104	100–108	97–102

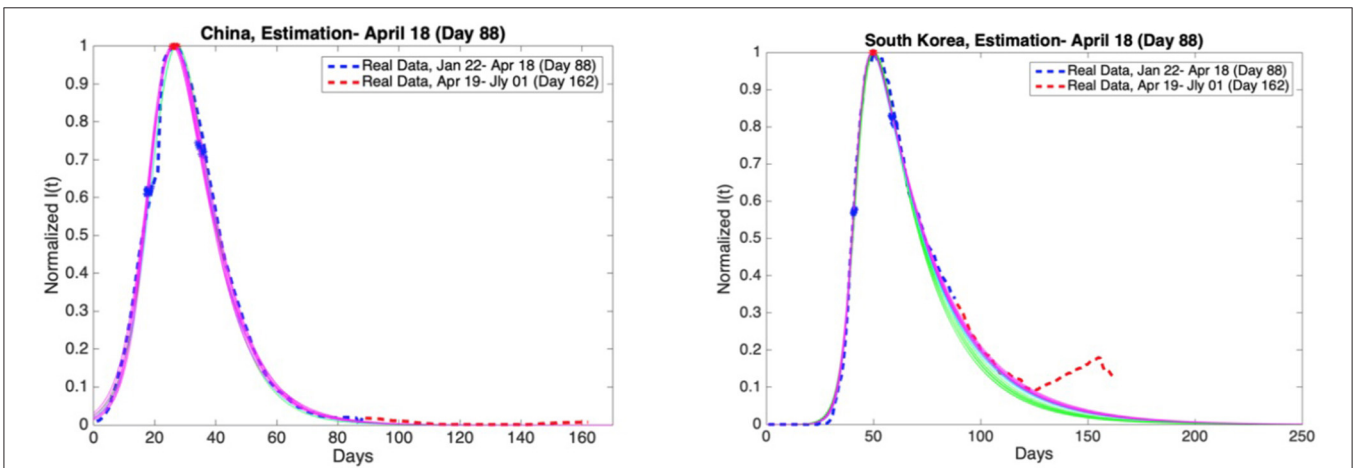


FIGURE 11 | China and South Korea: Graphs of estimation of normalized I(t) curves for the best 10 SIR models for each time span (blue dashed curve: real data till the day 88, red dashed curve: real data between the day 88 and day 162). Accuracy of estimates was ascertained through comparison with the real data post 18th April. Data is normalized by dividing with the maximum value of infectious cases between day 1 and day 88.

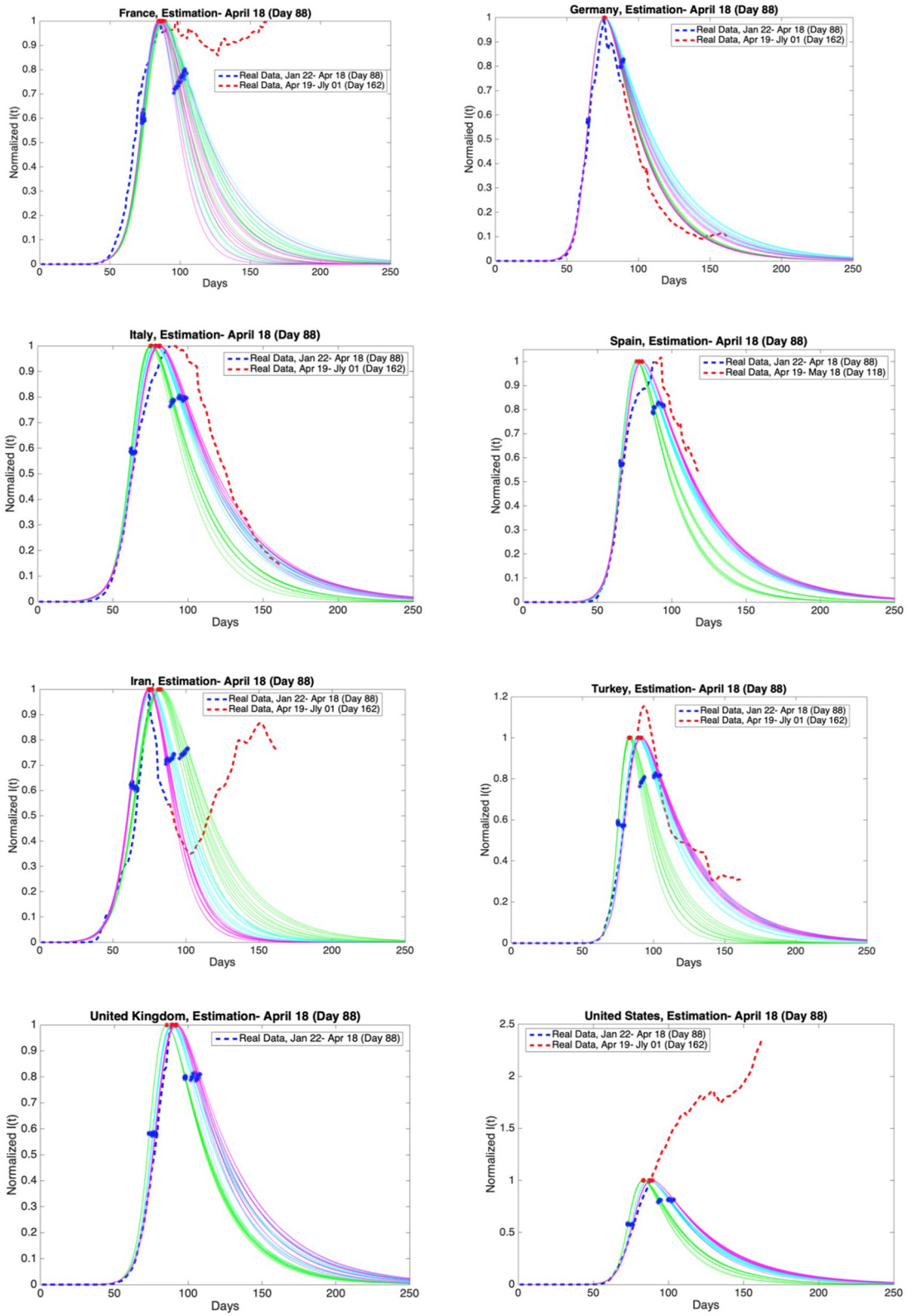


FIGURE 12 | Graphs of estimation of normalized I(t) curves for the best 10 SIR models for each time span (blue dashed curve: real data till the day 88, red dashed curve: real data between the day 88 and day 162 except Spain and the United Kingdom) and the countries France, Germany, Italy, Spain, Iran, Turkey, the United Kingdom, and the United States. Accuracy of estimates was ascertained through comparison with the real data post 18th April. Data is normalized by dividing with the maximum value of infectious cases between day 1 and day 88.

The estimates for Italy is consistent with the real data. On the other hand, the maximum of the infectious cases occurred slightly later than expected. In addition, Italy is going through the third and the fourth phases more slowly than expected. The most recent data conforms closely to our predictions.

Spain has not shared the data for daily discharged patients since the 19th of May (day 119). Therefore, the estimates are compared with the real data up to 18th May (day 118). Our estimates and the real data for Spain are consistent. However, the maximum of the infectious cases occurred slightly later than expected. In addition, Spain is going through the third and the fourth phases more slowly than expected as in Italy.

Our estimates and the real data for Turkey are consistent. The maximum of the infectious cases occurred slightly later than expected. The decrease in the active infectious cases was close to our estimates up to a certain date. Later, the number of infectious cases shows fluctuations. Loosening quarantine restrictions on 1st June may be the reason for these fluctuations.

United Kingdom has not shared the data for daily discharged patients for a long time. We can not compare our estimation with the real data.

As for the USA the spread of the epidemic has been beyond all predictions and it is still growing.

The discrepencies between estimates and real data and the failure to estimate parameteres for USA can be explained as follows. The basic reproduction number $\Re_0$ is beta/eta and beta is a product of the virulence of the virus and the contact rate in the society. The contact rate depence crucially on lock-down measures. As these measures change, the course of the epidemic follows a different dynamic.

CONCLUSION

The epidemic parameters of Covid-19 for 10 selected countries are estimated by using the data released by the state offices. These parameters include the basic reproduction number, mean duration of infectious period, the time at which the number of infectious cases reaches its maximum, the time at which the rate of increase in the number of infectious cases reaches its maximum, the time at which the rate of decrease in the number of infectious cases reaches its maximum. For each country, the best Susceptible-Infected-Removed (SIR) models fitting *cumulative case data* are obtained. A wide variety of intervals with different scales of the parameters, basic reproduction number $\Re_0$, and infectious period T, are observed. More specifically, the basic reproduction number and mean duration of infectious period are estimated only for China since the spread of the disease there is over. These parameters are found to be 3 and 5, respectively. The fact that the median incubation and infection periods are $\sim$5 days, supports the observations for $\Re_0$ and T. However, the basic reproduction number and infectious period for other countries cannot be predicted from the normalized data but the timing of key events can be estimated quite reliably. To summarize, we show that the quantity that can be the most robustly estimated from the normalized data, is the timing of the highest rate of increase in the number of infections, i.e., the inflection point of the number of infected individuals. However, it should be pointed out that the analysis performed by the SIR model for South Korea provides dissimilar results which can be explained by the unique age distribution nature of the confirmed cases.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: http://www.worldometers.info/coronavirus/; http://epikhas.khas.edu.tr/.

AUTHOR CONTRIBUTIONS

AB and AD performed the computations. AD collected the data. OE provided the medical insights. SA and AP-D performed the literature survey and wrote the paper. All authors contributed to the article and approved the submitted version.

REFERENCES

1. World Health Organization. *Coronavirus Disease 2019 (COVID-19): Situation Report*, 52. (2020). Available online at: https://www.who.int/emergencies/diseases/novel-coronavirus-2019/situation-reports (accessed April 27, 2020).
2. Tizzoni M, Bajardi P, Poletto C, Ramasco JJ, Balcan D, Gonçalves B, et al. Real-time numerical forecast of global epidemic spreading: case study of 2009 A/H1N1pdm. *BMC Med.* (2012) 10:165. doi: 10.1186/1741-7015-10-165
3. Fanelli D, Piazza F. Analysis and forecast of COVID-19 spreading in China, Italy and France. *Chaos Solitons Fractals.* (2020) 134:109761. doi: 10.1016/j.chaos.2020.109761
4. Kucharski AJ, Russell TW, Diamond C, Liu Y, Edmunds J, Funk S, et al. Early dynamics of transmission and control of COVID-19: a mathematical modelling study. *Lancet Infect Dis.* (2020) 20:553–8. doi: 10.1016/S1473-3099(20)30144-4
5. Roda WC, Varughese MB, Han D, Li MY. Why is it difficult to accurately predict the COVID-19 epidemic? *Infect Dis Model.* (2020) 5:271–81. doi: 10.1016/j.idm.2020.03.001
6. Roosa K, Lee Y, Luo R, Kirpich A, Rothenberg R, Hyman JM, et al. Real-time forecasts of the COVID-19 epidemic in China from February 5th to February 24th, 2020. *Infect Dis Model.* (2020) 5:256–63. doi: 10.1016/j.idm.2020.02.002
7. Li L, Yang Z, Dang Z, Meng C, Huang J, Meng H, et al. Propagation analysis and prediction of the COVID-19. *Infect Dis Model.* (2020) 5:282–92. doi: 10.1016/j.jdm.2020.03.002
8. Mangoni L, Pistilli M. *Epidemic Analysis of Covid-19 in Italy by Dynamical Modeling* SSRN. (2020). Available online at: https://ssrn.com/abstract=3567770 (accessed August 21, 2020).
9. Jia W, Han K, Song Y, Cao W, Wang S, Yang S, et al. Extended SIR prediction of the epidemics trend of COVID-19 in Italy and compared with Hunan, China. *Front Med.* (2020) 7:169. doi: 10.3389/fmed.2020.00169
10. Perc M, Miksić NG, Slavinec M, Stožer A. Forecasting COVID-19. *Front Phys.* (2020) 8:127. doi: 10.3389/fphys.2020.00127
11. Cassaro A, Pires LF. Can we predict the occurrence of covid-19 cases? Considerations using a simple model of growth. *Sci Total Environ.* (2020) 728:138834. doi: 10.1016/j.scitotenv.2020.138834
12. Zhang X, Ma R, Wang L. Predicting turning point, duration and attack rate of covid-19 outbreaks in major western countries. *Chaos Solitons Fractals.* (2020) 135:109829. doi: 10.1016/j.chaos.2020.109829
13. Danon L, Brooks-Pollock E, Bailey M, Keeling MJ. A spatial model of CoVID-19 transmission in England and Wales: early spread and peak timing. *medRxiv [Preprint]*. (2020). doi: 10.1101/2020.02.12.20022566

14. Batista M. Estimation of the final size of the COVID-19 epidemic. *medRxiv [Preprint]*. (2020). doi: 10.1101/2020.02.16.20023606
15. Hethcote HW. Qualitative analyses of communicable disease models. *Math Biosci.* (1976) 28:335–56. doi: 10.1016/0025-5564(76)90132-2
16. Bilge AH, Samanlioglu F, Ergonul O. On the uniqueness of epidemic models fitting a normalized curve of removed individuals. *J Math Biol.* (2015) 71:767–94. doi: 10.1007/s00285-014-0838-z
17. Shen M, Peng Z, Xiao Y, Zhang L. Modelling the epidemic trend of the 2019 novel coronavirus outbreak in China. *bioRxiv [Preprint]*. (2020). doi: 10.1101/2020.01.23.916726
18. Tang B, Wang X, Li Q, Bragazzi NL, Tang S, Xiao, et al. Estimation of the transmission risk of the 2019-nCoV and its implication for public health interventions. *J Clin Med.* (2020) 9:462. doi: 10.3390/jcm9020462
19. Read JM, Bridgen JR, Cummings DAT, Ho A, Jewell CP. Novel coronavirus 2019-nCoV: early estimation of epidemiological parameters and epidemic predictions. *medRxiv [Preprint]*. (2020). doi: 10.1101/2020.01.23.20018549
20. Zhao S, Lin Q, Ran J, Musa SS, Yang G, Wang W, et al. Preliminary estimation of the basic reproduction number of novel coronavirus (2019-nCoV) in China, from 2019 to 2020: a data-driven analysis in the early phase of the break. *Int J Infect Dis.* (2020) 92:214–7. doi: 10.1016/j.ijid.2020.01.050
21. Samanlioglu F, Bilge AH, Ergonul O. A susceptible-exposed-infected-removed (SEIR) model for the 2009-2010 A/H1N1 epidemic in Istanbul. *arXiv[Preprint]. arXiv:1205.2497* (2012).
22. Dobie AP, Demirci A, Bilge AH, Ahmetolan S. On the time shift phenomena in epidemic models. *arXiv[Preprint]. arXiv:1909.11317* (2019).
23. Bilge AH, Samanlioglu F. Determination of epidemic parameters from early phase fatality data: a case study of the 2009 A (H1N1) pandemic in Europe. *Int J Biomath.* (2018) 11:1850021. doi: 10.1142/S1793524518500213

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Public Health

ORIGINAL RESEARCH
published: 03 September 2020
doi: 10.3389/fpubh.2020.569669

Anticipating the Novel Coronavirus Disease (COVID-19) Pandemic

*Taranjot Kaur[1], Sukanta Sarkar[1], Sourangsu Chowdhury[2], Sudipta Kumar Sinha[3], Mohit Kumar Jolly[4] and Partha Sharathi Dutta[1]**

[1] Department of Mathematics, Indian Institute of Technology Ropar, Rupnagar, India, [2] Department of Atmospheric Chemistry, Max Planck Institute for Chemistry, Mainz, Germany, [3] Department of Chemistry, Indian Institute of Technology Ropar, Rupnagar, India, [4] Centre for BioSystems Science & Engineering, Indian Institute of Science, Bengaluru, India

The COVID-19 outbreak was first declared an international public health, and it was later deemed a pandemic. In most countries, the COVID-19 incidence curve rises sharply over a short period of time, suggesting a transition from a disease-free (or low-burden disease) equilibrium state to a sustained infected (or high-burden disease) state. Such a transition is often known to exhibit characteristics of "critical slowing down." Critical slowing down can be, in general, successfully detected using many statistical measures, such as variance, lag-1 autocorrelation, density ratio, and skewness. Here, we report an empirical test of this phenomena on the COVID-19 datasets of nine countries, including India, China, and the United States. For most of the datasets, increases in variance and autocorrelation predict the onset of a critical transition. Our analysis suggests two key features in predicting the COVID-19 incidence curve for a specific country: (a) the timing of strict social distancing and/or lockdown interventions implemented and (b) the fraction of a nation's population being affected by COVID-19 at that time. Furthermore, using satellite data of nitrogen dioxide as an indicator of lockdown efficacy, we found that countries where lockdown was implemented early and firmly have been successful in reducing COVID-19 spread. These results are essential for designing effective strategies to control the spread/resurgence of infectious pandemics.

Keywords: COVID-19, critical transitions, indicators of critical slowing down, social distancing policies, non-pharmaceutical interventions

OPEN ACCESS

Edited by:
Zisis Kozlakidis, International Agency for Research on Cancer (IARC), France

Reviewed by:
Sérvio Pontes Ribeiro, Universidade Federal de Ouro Preto, Brazil
Enahoro Iboi, Spelman College, United States

****Correspondence:***
Partha Sharathi Dutta
parthasharathi@iitrpr.ac.in

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Public Health

Received: *04 June 2020*
Accepted: *12 August 2020*
Published: *03 September 2020*

Citation:
Kaur T, Sarkar S, Chowdhury S, Sinha SK, Jolly MK and Dutta PS (2020) Anticipating the Novel Coronavirus Disease (COVID-19) Pandemic. Front. Public Health 8:569669. doi: 10.3389/fpubh.2020.569669

1. INTRODUCTION

The outbreak of the COVID-19 disease caused by a novel pathogenic coronavirus (SARS-CoV-2), which began in Wuhan, China, in December 2019, is a global challenge for the healthcare, economy and the society (1). The World Health Organization (WHO) assessed the epidemics of the disease (COVID-19) and declared it a Public Health Emergency of International Concern (PHEIC) (2). Since the Wuhan outbreak, nearly all the United Nations member countries have experienced a rapid spread of the virus and have been taking preventive measures to overcome the threats posed by the pandemic (3). Over the past years, several waves of viruses, such as influenza, cholera, and HIV have transmitted across the world to pose a significant threat to human health. Investigations on the interventions of these outbreaks have increased within the predictive theory of infectious diseases. Importantly, prior understanding of the epidemic spread of COVID-19 can provide an effective mitigation policy.

The COVID-19 disease can spread in a population through infected symptomatic/asymptomatic individuals who come into contact directly or indirectly (4). Concerned with the public health and well-being affected due to COVID-19, various countries have thus adopted comprehensive clinical and non-pharmaceutical strategies. The non-pharmaceutical interventions have included social distancing, such as the closure of schools, banning of large gatherings, isolation of symptomatic individuals, and monitoring of travelers, particularly those from COVID-19 hotspots (5–8). There also exists evidence of similar non-pharmaceutical interventions used to mitigate the 1918 influenza pandemic (9, 10). Evidence also highlights the importance of mitigation interventions in controlling the transmission of the SARS-CoV-2 virus (6, 11, 12). Nonetheless, the timing of the implementation of strategies varies between countries and can significantly influence the incidence curve of the epidemic (13).

The COVID-19 incidence curve of total confirmed cases for many countries initially demonstrates a gradual increase near the start of the epidemic and is often followed by a sudden shoot or a transition to a supercritical state (14–18), as the disease spreads (major outbreak due to human-to-human transmission). This sudden transition places a considerable burden on the limited availability of the public health resources required to treat the disease and inhibit its further spread. Most of the studies on sudden transitions concern catastrophic shifts associated with a saddle-node bifurcation; however, epidemic transitions are non-catastrophic and associated with a transcritical bifurcation (17, 19). In general, an epidemic transition occurs when the basic reproduction number (or $\mathcal{R}_0$) of the disease becomes >1 and a population moves from a subcritical to a supercritical state. In many countries, however, major outbreaks of COVID-19 did not initially occur, though the $\mathcal{R}_0$ of the disease is known to be more than one from the very beginning (20). In fact, this may be associated with a tipping delay where a population faces the first major outbreak at a higher value of $\mathcal{R}_0$ than one (14, 21), impending our ability to mitigate. It is thus crucial to anticipate this precarious transition to take effective controlling measures for the outbreak. There exists a rich history of investigations that can predict processes that could lead to ecological outbreaks (19, 22–24). Theory suggests applicability of a variety of leading generic indicators, widely known as Early Warning Signals (EWSs) (e.g., variance, autocorrelation, skewness, and kurtosis), to identify the proximity of a system to such a critical transition (22, 23, 25, 26). For instance, in time series data following ancient abrupt climate shifts, EWSs could be identified before the critical transition took place (27). Similarly, EWSs were seen in the resurgence of malaria in Kericho, Kenya (18).

EWSs are hallmarks of critical slowing down (CSD) of a system as it approaches a catastrophic/non-catastrophic transition. The phenomenon of CSD is caused by to the loss of resilience in the system such that even small disturbances can invoke an often irreversible transition to an alternative stable state (28–31). In particular, dynamical systems are continuously subject to shocks that may be extrinsic or intrinsic perturbations. In epidemiological theory, intrinsic perturbations can be determined by the pathogen's novelty in a new host, which may depend upon various health factors associated with the host. Furthermore, the mode of transmission, person-to-person contact, and number of imported cases may account for external perturbations for disease spread. Increased perturbations may drive a system far from its original state and can increase the time required for fluctuations in the number of cases to dampen. The system thus loses its resilience, as it may eventually diverge at a transition from a low burdened to a high burdened state. The phenomenon of CSD can be captured as a large time taken by a system to return to its previous states due to which the rate of return of a system decreases prior to a transition. Moreover, it leads to an increase in the short-term memory of a system, this feature can be identified by the changes in the correlation structure of a time series preceding a critical transition (22, 23, 26, 32, 33).

Model based epidemiological investigations predict the phenomenon of CSD preceded by the epidemic transitions (14, 15, 34). These studies are built on the applicability of CSD-based EWSs to anticipate disease emergence. However, construction of emerging disease models can be complicated partly due to non-linearity in many natural systems. Additionally, data availability of key epidemiological parameters, such as rate and mode of transmission, duration of infection, and the novelty of the pathogen in a new host, can pose a barrier toward disease predictive theory. The key support of CSD-based EWSs analyzes over modeling prediction is that it does not require comprehensive data calibration and can be calculated using observed data. Furthermore, it is studied that imperfections in the disease data does not form a barrier in applicability of EWSs (15).

To mitigate the epidemic, China strictly restricted public movement and followed with measures of quarantine and symptomatic isolation 24 days after (i.e., January 23) the arrival of the first reported case. The total reported cases (confirmed) at the time of the lockdown were nearly 623 (accounting for $\sim 4.4732\times10^{-7}$ of the total population). The daily increase in the number of confirmed cases in China was saturated in mid-March, hence flattening the incidence curve of the total confirmed cases. European countries adopted different non-pharmaceutical measures to intervene in the disease transmission. The spread began later in Italy compared to China; however, the strict interventions were initiated on March 9, which marks a gap of nearly 40 days from the first reported case with $\approx 1.22 \times 10^{-4}$ proportion of the cases. Spain, which is continued to suffer severely by the virus, reported its first infected case on February 1 and took nearly 45 days when the proportion of affected cases was more than 9.05×10^{-5}, to put the country into lockdown (see **Table S1** in **Supplementary Material**). India confirmed its first case on January 30 and prompted a "Janata curfew" on March 22 followed by a nationwide lockdown on March 25 for complete cessation of public contacts (nearly 55 days after the first case being reported). The proportion of cases were $\sim 2.36 \times 10^{-7}$ of its population (COVID-19-infected cases), while this proportion was more than 1.7×10^{-4} in the US. Therefore, it is essential to understand how prolonged gaps between the arrival of the epidemic and non-pharmaceutical interventions, such as quarantining/social distancing can influence public health and

the environment at a national as well as a global scale. Of greater interest is outlining whether the EWSs can be useful to stifle the spread of an epidemic.

In this work, we analyze how the timing of strict controlling strategies influence the COVID-19 incidence curve of the total confirmed cases in different countries. We first use the "change in the gradient" analysis (for details see **Section 4: Detection of the Transition Phase** in **Supplementary Material**), to estimate the emergence of the transition phase in incidence curves. The occurrence of CSD is then analyzed using the data prior to the transition. We calculate the variance and lag-1 autocorrelation function of the time series data of the cumulative confirmed cases each in nine different countries. Our work suggests that the dynamics of incidence curve in the initial days (depending upon the country), since the first reported case, can signal an upcoming sudden rise in the cumulative number of infected cases. Preliminary intervention is thus crucial for an effective and timely containment of the disease emergence or resurgence. Delay in the strict surveillance and control measures can increase the time to contain the spread, which in turn will affect a larger proportion of the population. Furthermore, the proportion of the affected cases on the commencement of public health measures plays a significant role in containing the epidemic in each country. The time gap of implementation of interventions from the arrival of the first case is almost similar for many countries, such as Italy, India and Germany. However, the EWSs depict an upcoming rise in Italy and Germany relatively earlier than in India. The relatively low proportion of the affected cases in the case of India compared to Italy or Germany can be a significant factor, explaining a slow rise for India but a relatively disruptive situation in the other countries. A combination of these two factors for India may thus restrict the extent of COVID-19 spread in the country, as compared to many other countries across the world. Importantly, despite keeping control of the situation up to April 29, India, having greater carrying capacity for the disease and several challenges to sanitization control (35), needs strict and highly effective interventions for continued suppression in the daily number of cases. We conclude that model-independent forecasting systems can be applied to clinical datasets for predictability of the disease re-occurrence and formulate control policies.

2. RESULTS

We obtain the datasets of the cumulative number of the COVID-19 cases from the date of reporting of the first affected person up to April 29, 2020, for India, China, South Korea, the United States (US), Singapore, Germany, Italy, the United Kingdom (UK), and Spain (for the data source see Materials and Methods). **Figure 1** depicts the incidence curve of the affected population in each of these countries. Interestingly, it is noted that the incidence curve of the confirmed cases follows a slow increment during initial time period ranging from ≈20 to 50 days for different countries, which can be interpreted as a time window to control the epidemic promptly and effectively. Since human-to-human contact is a leading transmitter of the disease, by-passing a certain threshold of infected cases, the incidence curve thus shows an increasing slope and finally depicts a transition in the number of infected cases (see **Figure 1**) (36). It is important to note that the growth in number of cases for China and South Korea, countries that initiated public monitoring/social distancing actions relatively earlier than the other countries, saturates after nearly 3–4 weeks from the initiation of the lockdown. The shift of the COVID-19 from a low-burden to a high-burden state can be associated with the phenomenon of critical transition. We thus employ statistical methods that can monitor the onset of the transition phase and provide insights into the incidence curve so as to suggest establishing worldwide disease elimination campaigns.

2.1. Signals of Critical Slowing Down

To estimate statistical indicators anticipating the upcoming shifts in each country, we consider the data of the cumulative daily number of COVID-19 cases before a transition is detected in the incidence curve of the epidemic (shaded regions in **Figure 1**) (for most of the countries, a transition threshold is detected by a gradient change analysis, for details see **Section 4: Detection of the Transition Phase** and **Figure S5** in **Supplementary Material**). To examine whether the system slows down to recover from perturbation while approaching the transition, we calculate the variance and autocorrelation at first lag [ACF(1)] of each extracted data for all the nine countries (see Materials and Methods). We have also calculated a few other generic EWSs of CSD, like density ratio, skewness, and kurtosis (for details see **Section 1: Early Warning Indicators** and **Figure S1** in **Supplementary Material**). CSD is reflected in systems near a critical transition through an increase in the variance and autocorrelation. We observe that the short-term memory of the time series data exhibits an increasing trend in most of the countries (**Figure 2**). However, there are no positive signals of CSD exhibited by ACF(1) for the datasets of India or Italy (**Figures 2J,P**). The increase in the variance forewarns a sudden rise in the number of the COVID-19 cases for these countries. Furthermore, the strength of the signals varies among countries depending upon the datasets determining the cumulative number of affected populations in individual countries. For instance, we observe a weak increase in variance in case of Singapore, and the trends in China and the US are observed to be very strong, with ACF(1) approaching close to 1 (see **Figures 2K,M**) (32). Since the time lag of up to almost 2 weeks is expected for the detection of symptomatic cases (37), the analyses suggest that the total cases gathered when the phenomenon of CSD is observed must have been infected with the disease around 2 weeks ago. Thus, early preventive and surveillance strategies can be capable of suppressing the severity of COVID-19 outbreak (38).

2.2. EWSs and Enforcement of Interventions

The timing of intervention measures varies among the countries. Notice that, apart from China and South Korea, in other countries, EWS analyses are carried out using the data before the

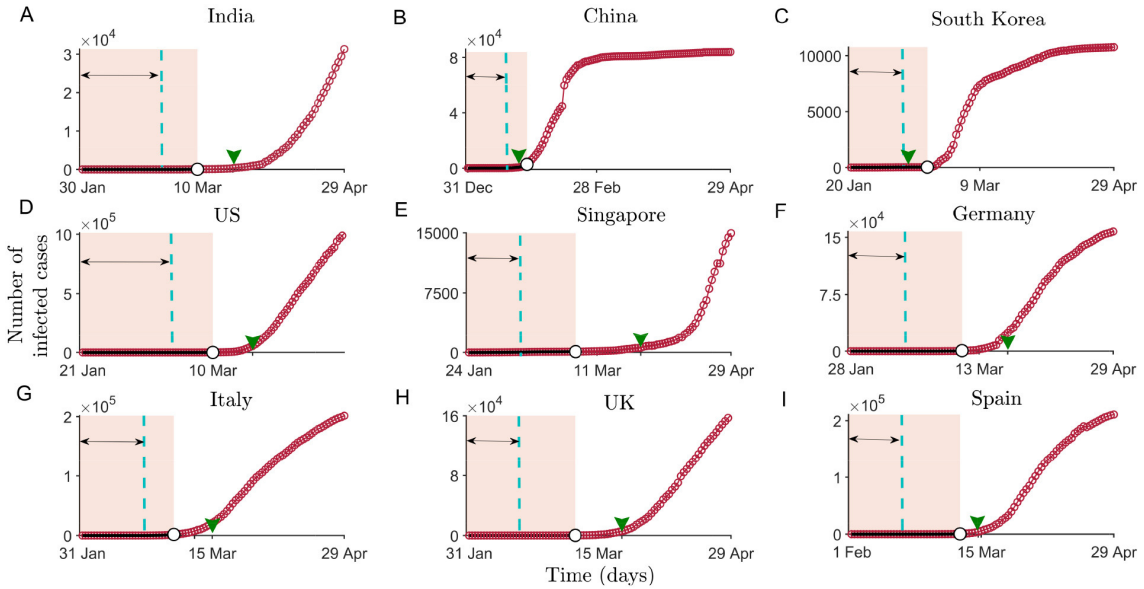


FIGURE 1 | Time series constructed as cumulative number of infected cases in nine different countries across the world from the onset of the epidemic in the respective countries up to April 29. **(A-I)**: Shaded regions depict the pre-transition phase for different countries from the onset and mark the data used to compute the indicators of critical slowing down. The double-sided arrow marks the size of the moving window (up to the vertical dashed line). In the subfigures, the arrowhead on the *x*-axis marks the beginning of the officially recorded social-distancing and/or lockdown dates (see **Table S1** in **Supplementary Material**).

implementation of social distancing measures. China was the first country to take the containment measures, nearly 24 days after the beginning of the epidemic, while Italy took around 40 days, and other countries followed even later. As a consequence, the COVID-19 incidence curve in China flattened after nearly 20–25 days of implementing the intervention measures. Like China, South Korea adopted different combinations of controlling measures around mid-February (in the time window of 20–25 days since the epidemic began there). This measure was accompanied by a drop in the number of cases, and the curve followed the pattern observed for China (**Figure 1C**). The rising indicators of CSD also suggest that the time gap in implementing the protocols, such as the closure of public gatherings, controlled public movement, and lockdown, can significantly influence the incidence curve and result in the extended time required to flatten it. However, the interventions around 2–3 weeks prior to the change in the correlation pattern as well as variance in each of these countries can slowly hamper the daily increase in the number of cases.

The scenario is quite different in the case of India. The EWSs weakly signal the behavior of CSD within the initial 40 days of the disease emergence (**Figures 2A,J**). Due to a rise in the number of daily cases, we also analyze the EWSs in the incidence curve for India considering the cumulative number of infected cases of up to the beginning of the nationwide lockdown (March 25, **Figure 3A**) since the reporting of the first case. Here, we observe increasing trend in each of the generic indicators capable of capturing the phenomenon of CSD. The variance, autocorrelation, skewness, and kurtosis captures the strong signals of CSD (**Figures 3B–E**).

2.3. Onset of Social Distancing Practices and the Affected Population Density

Another important aspect is to consider the reported proportion of a population affected at the time of the implementation of intervention measures. So far, Germany, which accounted for one of the largest outbreaks in Europe around mid-March, had visible signals of the forthcoming transition (**Figures 2F,O**). It is noted that each of the countries, namely India, Germany, and Italy, adopted concerned public health measures around the time when the EWSs were visible in their respective datasets (see the arrowheads on the *x*-axis in **Figure 1**). However, the fraction of the population affected by that time in Germany and Italy was much higher ($\sim 2.9 \times 10^{-4}$ and 1.2×10^{-4}, respectively) compared to India $- 2.36 \times 10^{-7}$. Thus, the incidence curve projected a significant rise in these two countries, whereas the rise in the number of cases in India is relatively slow and is expected to follow a similar response owing to the effectiveness of these interventions. Overall, our analyzes suggest that delayed interventions (depending upon the signals of CSD) along with the fraction of the affected population can influence the country-wide variation in the daily number of rising cases.

2.4. Sensitivity Analysis of the Generic Indicators

The choices made to remove/filter out non-stationarities in the time series datasets using Gaussian detrending can also influence the trends observed. Thus, it is necessary to test the robustness of the estimated trends toward the choice of rolling window size and the filtering bandwidth. Here, we employ sensitivity analysis for the variance (see **Figure 4**) and ACF(1)

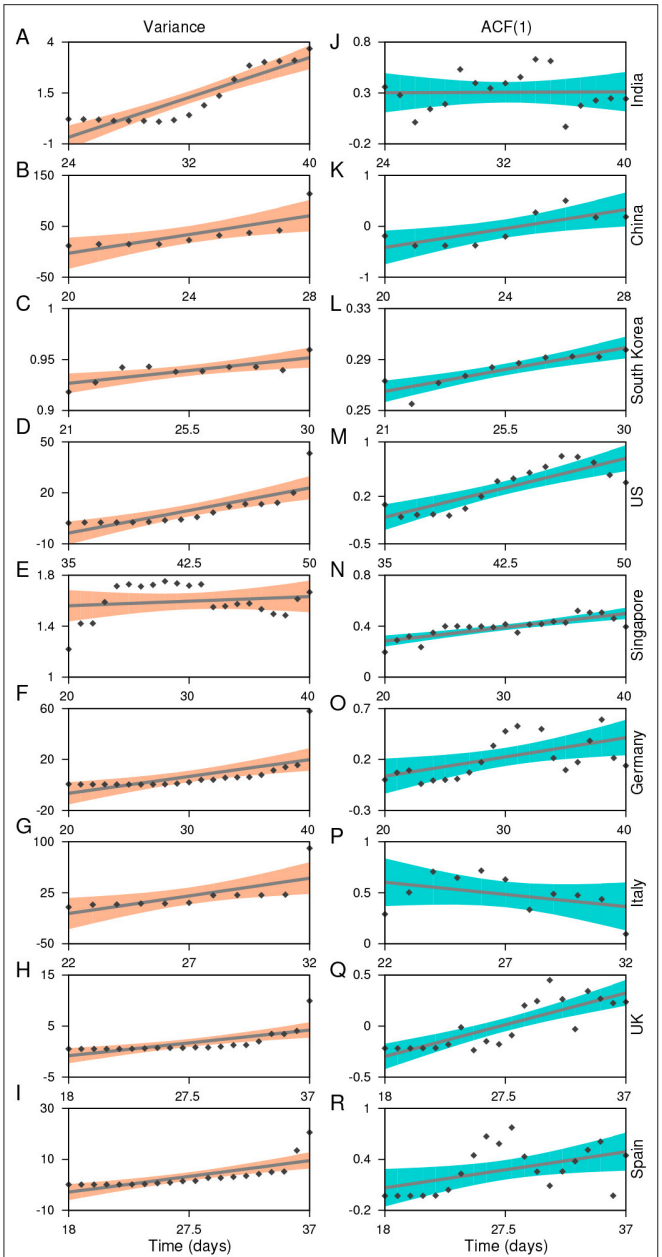


FIGURE 2 | Statistical estimates used to analyze the signals of a forthcoming transition in the COVID-19 incidence curve. **(A-I)**: Figures on the left panel depicts the variance each for India, China, South Korea, USA, Singapore, Germany, Italy, UK, and Spain. **(J-R)**: The right panel shows the lag-1 autocorrelation of the time series data analyzed in the corresponding countries. Scattered points are the estimated values of the respective slowing down indicators. Solid lines reflect the increasing/decreasing trend in the indicators and are obtained by fitting linear regression models. The shaded regions are the confidence bounds for the fitted models.

(see **Figure 5**) using the CSD dataset. Sensitivity analysis ease outs to disentangle accurate signals of an impending transition from the false ones for a wide range of window sizes and bandwidths. We use Kendall-τ estimates of these indicators for all the combinations of these two parameters (for details, see Materials and Methods). Furthermore, we test the sensitivity of

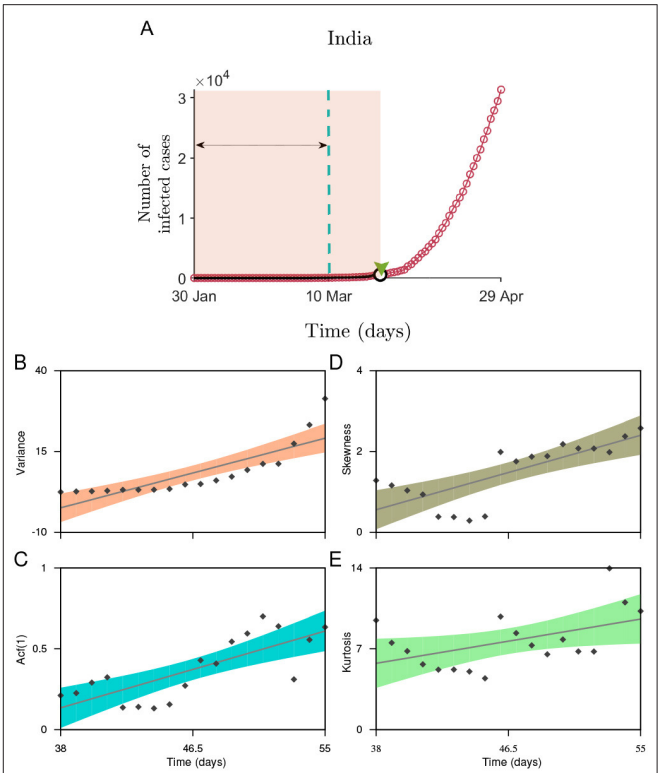


FIGURE 3 | Statistical analysis to measure the indicators of CSD for the dataset of India up to the date of the nationwide lockdown. **(A)** The incidence curve depicting the fraction of people infected from January 30 up to April 29. The shaded region is the data used to calculate the changes in the generic indicators. The arrow marks the size of the rolling window used to calculate the statistical signals. The estimated values of the slowing down indicators **(B)** variance, **(C)** autocorrelation function at lag-1, **(D)** skewness, and **(E)** kurtosis. Solid lines are the fitted linear regression models to analyze the trend in the indicators along with the confidence bounds (shaded regions).

these parameters on the *P* values of the estimated indicators (for details see **Section 3: Sensitivity Analysis** and **Figures S2,S3** in **Supplementary Material**).

We find that the observed trends in the variance are robust to the choice of parameters and does not vary between the datasets of most countries. High bandwidths reveal the opposite outcome of the variance for the datasets of South Korea (**Figures 4C,L**) and Singapore (**Figures 4E,F**). Since we use the bandwidth, which gives the best fit and does not over-fit or under-fit the data therefore, the choice of window size can influence the observed trends. In our work, we find a large size of rolling window can alter the EWSs analysis and misleading estimates for the autocorrelation function (**Figures 5A–N**). False signals of an alarming situation can deviate from understanding the gravity of any situation and intensity of surveillance needed. Thus, the choice of these parameters is crucial in anticipating the signals of a forthcoming transition and implementing convincing public health measures.

2.5. Surrogate Analysis

The lower number of data points available for the analysis can lead to feeble trends and influence the probability of

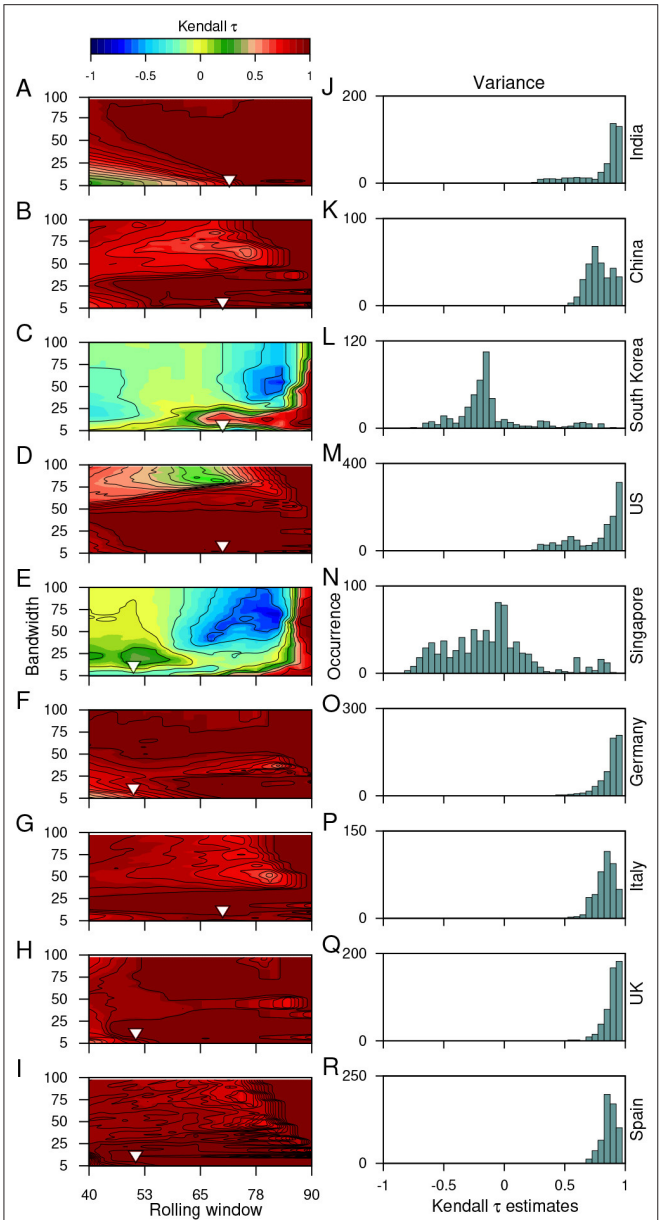


FIGURE 4 | The sensitivity of the choice of the rolling window size and the filtering bandwidth to estimate the EWSs. **(A-I)**: Contour plots demonstrate the effect of moving window size and the filtering bandwidth on the trends observed while calculating the changes in the variance of the time series, using the Kendall-τ test statistic. **(J-R)**: Panels on right show the frequency distribution of the trend statistic. The inverted arrows mark the choice of the filtering bandwidth and moving window size used to capture the trends in the variance of the time series data.

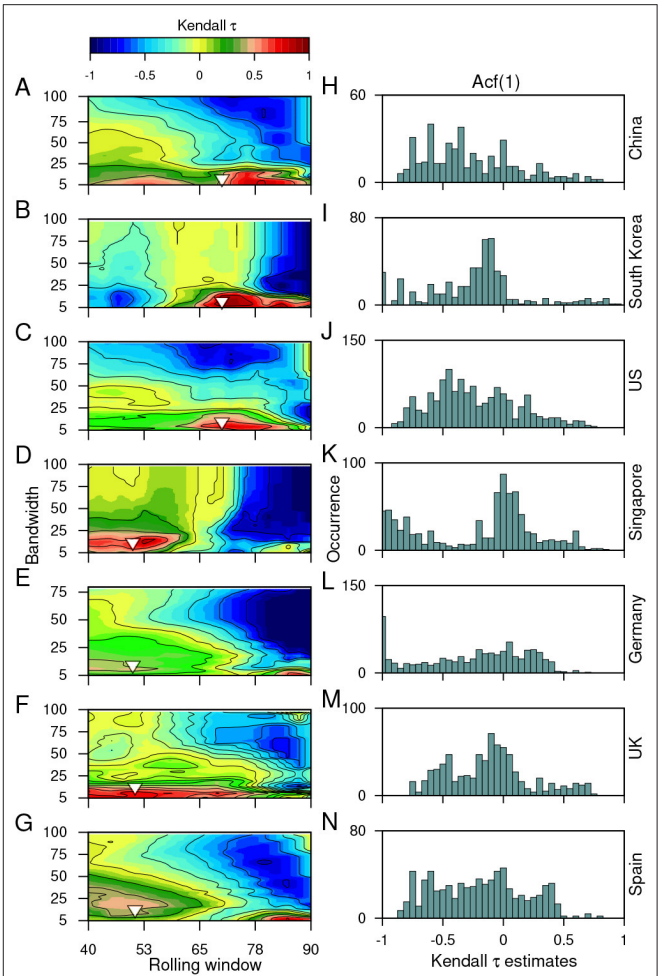


FIGURE 5 | The sensitivity of the choice of the rolling window size and the filtering bandwidth to estimate the EWSs. **(A-G)**: Contour plots demonstrate the effect of moving window size and the filtering bandwidth on the trends observed while calculating the short term correlation pattern using ACF(1) of the time series data, using the Kendall-τ test statistic. **(H-N)**: Panels on right show the frequency distribution of the trend statistic. The inverted triangles mark the choice of the filtering bandwidth and moving window size used to capture the trends in the ACF(1) of the time series data.

occurrence of the increased signals of CSD by chance. Further, due to undocumented patients, there is always a chance of stochasticity in the number of reported cases. Thus, we studied the likelihood of coincidence in the occurrence of trends in the variance and the ACF(1) observed in our original datasets by investigating the indicators in the surrogate time series (see Materials and Methods). The surrogate time series is generated to follow similar distribution (mean and variance) of the data time series before the episode of a sudden rise in the number, denoted by shaded regions in **Figure 1** (see Materials and Methods). **Figure 6** depicts the distribution of the test statistic of the surrogate time series. Solid lines show the trend estimate obtained for the original time series. We calculate the probability of randomness of our observed estimates as the fraction of 1,000 surrogate time series having trend statistic of same or higher values than the original trend, i.e., $P(\tau^* \leq \tau)$. The probability of, by chance, obtaining similar trend statistic varies from country to country, depicting significant estimates for changes in the variance, except for South Korea (**Figures 6B,I**) and Singapore (**Figures 6D,K**). In the case of the US, however, the probability of randomness in our observed estimates is lower (**Figures 6C,J**), and rapid spreading in the

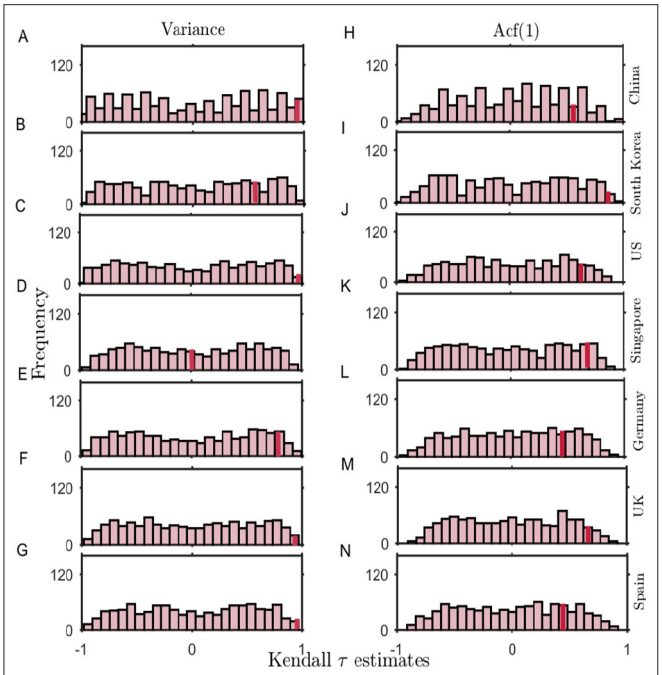


FIGURE 6 | The probability distribution of Kendall-τ test statistic on a set of 1,000 surrogate time series generated by bootstrapping and shuffling (with replacement) the residual time series of the original data. **(A-G)**: Histograms depict the distribution of the test statistic for the surrogate time series variance (left panels) and **(H-N)**: autocorrelation function at lag-1 (right panels). Solid lines indicate the limit beyond which the Kendall-τ of the surrogate data is higher than the statistic observed in the ACF(1) of the original time series.

TABLE 1 | Probability of, by chance, obtaining the observed trend statistic of the original data for the set of 1, 000 surrogates having similar distribution (mean and variance) as the original datasets.

Country	Kendall-τ (variance)	Kendall-τ [ACF(1)]
China	0.04	0.09
South Korea	0.21	0.01
US	0.001	0.1
Singapore	0.48	0.09
Germany	0.08	0.20
UK	0.001	0.05
Spain	0.001	0.21

The likelihood of randomness in the estimated variance and ACF(1) is mentioned for the datasets of each country studied in the work.

epidemic makes it keystone to consider applicability of EWSs to warn-off such events. The probability estimates P obtained by bootstrapping the datasets for each of the countries are given in **Table 1**.

Overall, we find a low probability of randomness in both the ACF(1) and the variance estimates for most of the cases. However, the observations are more significant for the variance. This analysis suggests the robustness of the variance as an EWS in predicting the signals of CSD.

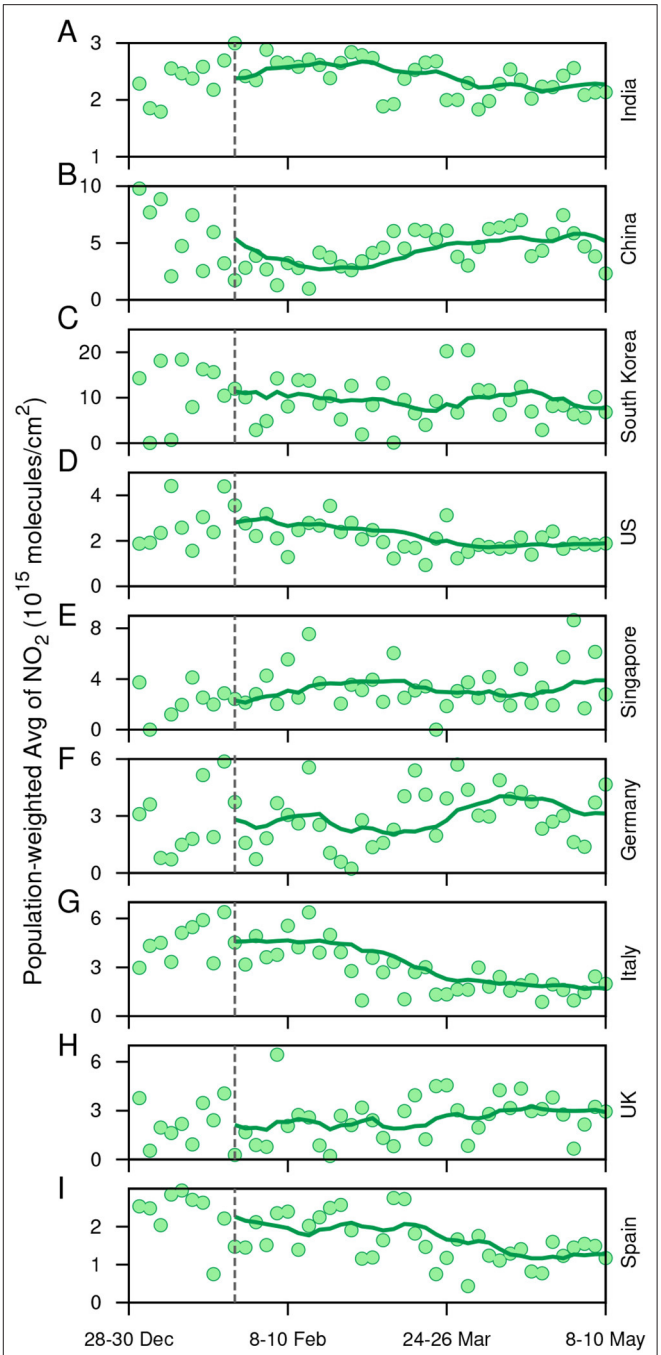


FIGURE 7 | (A-I): Time series of triads of the population-weighted total-column NO_2 (molecules/cm^2) density over the length of the study period for the nine countries considered in this work (depicted by the circular points). The solid curve in each subfigure represents a 10-triad moving window average of the time series.

2.6. Impact of COVID-19 Spread on the Atmospheric Total-Column NO_2 Density

The rigor of social distancing/intervention strategies can be measured by atmospheric data, as the lockdown periods have

witnessed better air quality across the globe (39). We note that anthropogenic NO_2 is emitted predominantly at the surface from transportation activities, industries, and power plants. NO_2 emitted has a short lifetime and can be transported up to a few hundred meters during the day. Therefore, NO_2 is expected to be a profound indicator of the efficiency of lockdown measures enforced by the countries. Thus, we first obtain time series of triads of the population-weighted total-column NO_2 (molecules/cm^2) density over the length of the study period for the nine countries considered in this work (the circular points in **Figure 7**) (for the NO_2 data source see Materials and Methods). The solid curve in each subfigure of **Figure 7** represents a 10-triad moving window average. In the majority of the countries, the timing of NO_2 decline concurs with the spread of the virus and the onset of pragmatic lockdown in a country may be hypothesized by the reversal (or break) in the trend of NO_2. In China (**Figure 7B**), the decreasing trend in NO_2 is evident from January to February; after that, it starts increasing which is coincident with the dynamics of the spread of COVID-19 disease. In India (**Figure 7A**), South Korea (**Figure 7C**), US (**Figure 7D**), Italy (**Figure 7G**), and Spain (**Figure 7F**), the decreasing trend in NO_2 coincides with time of the rapid spread in the virus (**Figure 1**). We estimate that after the date of official enforcement of lockdown, the time-averaged NO_2 decreased by 26.6% in China and 55.6% in Italy compared to the pre-lockdown period. Spain, USA, and India have also seen a significant decrease after the lockdown was enforced in these countries by 33, 22.9, and 11.8%, respectively. It increased in the UK and Germany by 18 and 32%, respectively, however, even after the initiation of lockdown, which indicates an inefficient closure of anthropogenic activities (like road and rail transport, industries, and power plants). The spatial distribution of total-column NO_2 for all the triads from Dec 28, 2019, to May, 10, 2020, can be visualized **Movie 1** in **Supplementary Material**. It should be noted that we did not control for meteorological variations, which may have a significant impact on total-column NO_2 over the period of our study (40). Overall, amidst the fears of the novel coronavirus, the countries where the lockdown intervened are expecting a rejuvenated environment. However, at the same time, possibilities of decreasing air pollutants when the world is not facing such harsh conditions is also important to understand.

2.7. A Minimal Stochastic Model

We propose a minimal kinetic model for the short-term prediction of the spreading of COVID-19 disease. Suppose that the only processes are infection and recovery. The processes can be described as

$$I + H \xrightarrow{k_i} I + I; \quad \text{and} \quad I \xrightarrow{k_r} H, \tag{1}$$

where I and H are infected and healthy people, respectively, and k_i and k_r are rate constants for infection and recovery. The first equation shows that if I is the infected people, then H becomes I at a rate k_i; and the second equation indicates that I recovers at a rate k_r. A minimal kinetic model can be formulated as ordinary differential equations for the population of I:

$$\frac{dI}{dt} = k_i I(1 - \frac{I}{K}) - k_r I, \tag{2}$$

where K is the size of the population.

We develop a master equation for the infected population by considering the two elementary processes (Equation 1). The transition probability at which the number of infected population increases from i to (i+1) is $w(i+1|i) = k_i i(1 - i/K)$, and the rate at which the number of infected population reduces from i to (i-1) is $w(i-1|i) = k_r i$. From these, the probability of finding i infectives in the system at time t, $P(i,t)$ can be obtained from the following equation:

$$\begin{aligned}\frac{dP(i,t)}{dt} &= w(i|i-1)P(i-1,t) + w(i|i+1)P(i+1,t) \\ &\quad -(w(i+1|i) + w(i-1|i))P(i,t).\end{aligned} \tag{3}$$

The above probabilistic model is solved by the kinetic Monte Carlo simulations by means of the Gillespie algorithm, which incorporates the intrinsic noise (41). The algorithm considers each of the events as individual realizations of the Markov process. The time and species numbers are updated stochastically by choosing the random processes.

To simulate the system (Equation 3), we first obtain the parameters from the cumulative time series data of confirmed cases for India, China, and South Korea. In the datasets, we fitted the below logistic function (which is a solution of Equation 2):

$$f(t) = \frac{a}{1 + b\exp(-ct)}, \tag{4}$$

where a, b, and c are parameters. Once we obtain these parameters for an individual country, we map them to our model and find the system parameters k_i, k_r and K, and i_0 is the initial infected population. We list those parameters below:

Country	k_i	k_r	K	i_0
India	0.608	0.486	11,722,830	2
China	1.235	0.988	419,880	27
S. Korea	0.9075	0.726	54,200	4

Then the above parameters are used to solve the Master equation (Equation 3), and we perform Monte-Carlo simulation to get stochastic trajectories up to April 15. We present the simulated stochastic trajectories in **Figure 8**. For each country, we have five trajectories. For China and South Korea, we find that our stochastic trajectories are consistent with the real time series of the number of infected people. However, for India, our result shows that on May 20, 2020, the number of infected people reached ∼109,262 (an average of final values of the five simulated trajectories).

The problem of predicting the spreading of COVID-19 is a complex one and depends on many factors like social distancing, an early detection of the disease, the detection of major hubs of the disease, etc. Here, we have provided a minimal kinetic model that uses the trends of the available data and may work only for short term prediction.

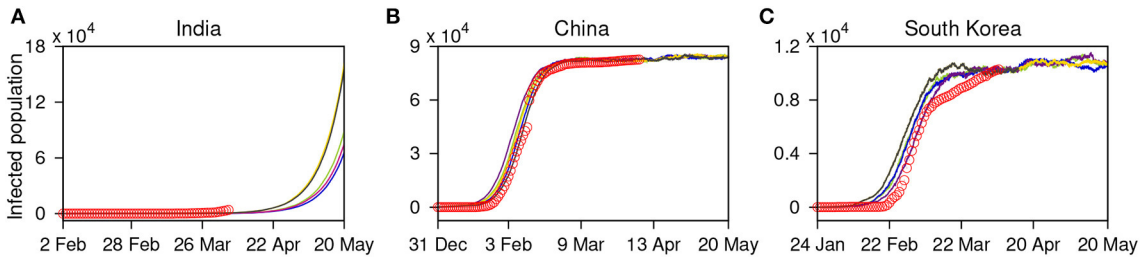


FIGURE 8 | Stochastic trajectories (marked with pink, yellow, gray, green, and blue curves) of the infected population generated using the Gillespie algorithm for: **(A)** India, **(B)** China, and **(C)** South Korea. The original datasets up to April 6 for the respective countries are depicted by circular (red) points for a comparison.

3. DISCUSSION

The COVID-19 pandemic revealed an exponential rise in the reported number of cases and has affected the public health, ranging from mild to severe conditions. Countries across the world are combating the spread of the coronavirus through various social distancing/intervention measures, such as the closure of schools and universities, banning of public events and large gatherings, isolation of symptomatic COVID-19 cases, implementation of mass quarantines, etc. For national as well as international control of public health, it is crucial to understand the significance of the onset timing of such measures (42).

The World Health Organization lately reported new cases being detected in several new countries across the globe (43–45). Our study can provide insight to tackle the ongoing pandemic and its associated incidence curve in the context of the timing and strength of the interventions. We use the data of the number of COVID-19 cases in nine different countries to investigate some statistical patterns in the incidence curves. The number of cases covers a small fraction of the population during the initiation of the epidemic, and the fraction remains nearly stagnant ranging nearly from 20 to 50 days from the arrival of the first case. Furthermore, the number of cases are increasing rapidly, and in a relatively shorter span, a significant fraction of the population can be affected. This trend is analogous to the idea that the incidence curve remains close to one stable state for a sufficient time and, crossing a time threshold, invokes a sudden shift/transition to another stable state, where a significant fraction of the population gets affected. In our work, we employ statistical indicators of critical slowing down to check if such transitions can be signaled beforehand and how the anticipation of such transitions can help mitigate such a crisis at a policy level.

We observe that the initial time window from the arrival of the first case in each country signaled an impending transition. An increase in the ACF(1) of the data as well as variance, before an actual rise in the number of cases, indicates the phenomenon of critical slowing down. Our work suggests that while non-pharmaceutical interventions are necessary to mitigate such an epidemic, the timing of initiation of concerned actions can strongly influence the outcome of the situation. Owing to the time lag in the detection of symptomatic cases, the statistical indicators suggest that a time period of 2–3 weeks before an impending transition is crucial to suppress the loss of public health. The controlled response of the epidemic incidence curve for China and South Korea can be associated with the time distance between implementation of interventions and the transition point. Both these countries initiated interventions before the visible signals of CSD in the incidence curve. Timely interventions were thus important factors to suppress the fluctuations in the number of cases and shape the curve. The analysis of EWSs analysis is crucial while defining the onset of the interventions and suppress the rise in daily cases. Importantly, another crucial aspect is the proportion of affected cases in each country, i.e., a measure of the fraction of the country's population, and not the absolute numbers, which is infected at the time of interventions, such as a strict lockdown. As probability of the propagation of disease can be thought of as mostly similar or equal amongst individuals across the globe, it depends upon the fraction of infected cases in each country during the beginning of interventions. For instance, the EWS analysis anticipated the upcoming rise in the incidence curves for both India as well as Italy, and, interestingly, both the countries imposed individual nationwide lockdown near the situation close to the transition (see **Table S1** in **Supplementary Material**). However, the control in India depicts better results in altering the incidence curve than that in Italy. The alterations in the incidence curve is most likely to be a consequence of a difference in the proportion of cases affected by the epidemic at the beginning of mitigation strategies. India, resembling China in terms of the total population density, accounted for $\sim 2.36 \times 10^{-7}$ cases of the total population, while Italy, with a relatively smaller population density, crossed 1.22×10^{-4} cases of their total population. Thus, even with imposition of the public health measures near the signals of CSD, the outcome for both the countries can vary dramatically. The variation is the consequence of the proportion of affected cases when visible signals of EWSs are observed and at the time of interventions. This suggests that the proportion of affected population during visible signals of CSD is key to shaping the disease incidence curve. The strength of the signals can alter the duration and scale of the interventions needed. Furthermore, the disruptive situation in the US is indicated by EWSs, as the EWSs indicators show significant trends for the US in addition to a large fraction of population being affected at that time. A sharp rise in the number of cases for the country is a consequence of both the delay in effective social distancing interventions as well as a significant proportion of affected cases. Overall, our work

suggests that, in almost all the countries, an imminent sharp rise in the incidence curve can be seen using statistical measures prior to the actual transition.

Another issue the infectious coronavirus raises is the quality of air pollution in countries where social distancing/lockdown is enforced. NO_2, which is majorly emitted from anthropogenic activities like land transportation, industries, and energy sectors, was estimated to decrease in consequence to lockdown measures implemented by the government of respective countries. Population-weighted average column NO_2 was found to decrease with amplification in a number of cases across most of the countries. Apart from this, NO_2 column quantities may be used as a proxy to estimate the effectiveness of a lockdown on air quality. We find that NO_2 column quantities started following a decreasing trend during the last week of February in Italy and the US, which indicates a partial unofficial closure of anthropogenic activities, taking into consideration that the official COVID-19-induced lockdown was enforced on March 09 and around March 25 in Italy and the US, respectively. In the UK, however, an increasing trend in the NO_2 column up until May 10 indicates no such public awareness to restrict anthropogenic activities (the government declared the lockdown from March 23). We acknowledge that the reduction in NO_2 is also associated with the compliance of the population of the individual nation to abide by the lockdown measures.

Furthermore, we suggest that the interventions employed by India may not come at a time when the curve is very far from reaching the transition; however, the smaller number of affected cases may be the determining factor in limiting the disease spread in India. Implementation of a nationwide lockdown in India may have better prepared the country for taking measures to control the epidemic spread and bend of the curve. However, our analysis also suggests that the period beyond the signals of CSD also needs efficient monitoring. The results of our minimal stochastic model predicted that, on May 20, the number of infected people could go up to ∼109,262. Thus, an extended period of such measures is needed and likely to be effective (6).

We envision that it is fundamental to identify the situation of such a crisis across the world and make use of the lead time. The EWSs can keep track of the changes in the trend statistics in the number of reported cases and warn when a threshold is reached. The statistical tools used can be beneficial to identify whether the features of shift in a system are suppressed by the intervention strategies being adopted. In particular, while different combinations of strategies are adopted to overcome such a crisis, the information of an upcoming transition and its threshold is important to formulate the degree of such interventions. However, special care should be taken in the choice of rolling window size and the filtering bandwidth while estimating the signals of slowing down. Inappropriate choices may give weak and/or diminished signals of an imminent transition, which may deviate from understanding the urgency of the situation. Another aspect to consider is that the varying extent of testing for COVID-19 across the countries may have affected the total number of reported cases; thus, our results here hold specifically for the number of reported cases.

4. MATERIALS AND METHODS

4.1. The COVID-19 Data Source

We have used the COVID-19 dataset provided by the European Centre for Disease Prevention and Control (ECDC): An agency of the European Union (available from https://www.ecdc.europa.eu/en/publications-data). Initially, we extract the data of the daily number of reported cases up to March 25, 2020, and in general mark the first date of the reported cases as the day of the beginning of the epidemic in the respective countries. Regardless of the affected person recovers or dies, the virus contraction occurs once; we thus consider cumulative data of the daily number of the confirmed cases for nine different countries for our study.

4.2. Data Selection

We use the available time series to test the predictability of an upcoming transition for each country. The generic indicators are examined using the time series segments before the transition in the number of cases of the epidemic (see **Section 4: Detection of the Transition Phase** and **Figure S5** in **Supplementary Material**) in each country (shaded regions in **Figure 1**).

4.3. Detrending

Often, non-stationarities in the data lead to false indications of impending transitions. To overcome this, we obtain the residual time series by subtracting a Gaussian kernel smoothing function from the empirical time series (23). Furthermore, we estimate the variance and autocorrelation at first lag for the residual time series choosing a rolling window size from the sensitivity analyzes of the time series data for each country. We choose the filtering bandwidth and avoided any under-fit or over-fit (for details see **Table S2** in **Supplementary Material**).

4.4. Autocorrelation at First Lag and Cariance

The fluctuations in the time series reveal different novel phenomena, such as sudden transition, flickering, stochastic switching, etc. It is established that followed by a perturbation, the rate of return of the system slows down near an impending transition or a tipping point. This phenomenon of slow return rate or recovery from a perturbation in the vicinity of a sudden transition is known as critical slowing down (CSD). We capture the signals of CSD by estimating changes in the short-term autocorrelation (at lag-1) and variance of the time series. CSD increases the short-term memory of the time series, which is observed through the correlation structure of the time series before a transition. We compute autocorrelation at lag-1 by fitting an autoregressive model of order 1 (of the form $z_{t+1} = \alpha_1 z_t + \epsilon_t$) using an ordinary least-squares fitting method. The time series analysis has been performed using the "Early Warning Signals Toolbox" (http://www.early-warning-signals.org/).

4.5. Sensitivity Analysis

The predictability of each of the indicator depends upon the datasets investigated as well as the choices made for processing

the data. Thus, it is essential to check the efficacy of our results to such choices. In particular, we analyze the sensitivity of our observations to the choice of rolling window size and degree of smoothing (filtering bandwidth) used during the calculation of indicators and detrending/filtering the datasets, respectively. We estimate the CSD indicators using window sizes ranging from 40 to 90% of the time series length in an increment of 1 point and for bandwidths ranging from 5 to 100% with the increment of 1 point. We quantify the robustness of the outcomes toward the range of window sizes and bandwidth using the distribution of the Kendall-τ test statistic.

4.6. Surrogates

To test the significance of our statistical analysis, we estimate Kendall rank correlation-τ test statistics for both the generic indicators. We generate 1,000 surrogate time series of the same length as the analyzed real datasets to test the likelihood of obtaining the computed trends by chance. The surrogate records are obtained on bootstrapping the real datasets by shuffling the original residual time series and sampling the data with replacement. This method generates the surrogate time series with a similar distribution of the original time series (27). For each surrogate, we consider the Kendall-τ estimate as the test statistic to measure the robustness of the outcomes. Furthermore, we calculate the fraction of the surrogates having the same or higher test static value than the original data and measure the probability $P(\tau^* \leq \tau)$ to calculate that the observed test statistic is by chance. We also generate surrogate time series using phase randomization method (for details see **Section 3: Surrogate Analysis** and **Figure S4** in **Supplementary Material**).

4.7. Satellite Retrieved Total Column NO_2

Worldwide, the lockdown response to the onset and spread of COVID-19 caused a decrease in daily and economic activities, which in turn is expected to cause a reduction in ambient air pollution. This can also be used as an indicator to determine whether government policies of lockdowns/restricted human movements are successful or not. To further examine this, we use the Ozone Monitoring Instrument (OMI) retrieved total column NO_2 (available from https://aura.gsfc.nasa.gov/omi.html) as a proxy to infer the change in anthropogenic air pollution for the time-period of our study. OMI flies onboard the EOS Aura sun-synchronous polar-orbiting satellite. It has a swath length of 2,600 km and a level-2 and spatial resolution of 13×24 km^2 (46). The OMI NO_2 column was satisfactorily validated against surface spectrometer measurements in recent studies (47, 48). To roughly obtain a global coverage, we consider 3-days time slices (triads) within which the overlapping swath overpasses were averaged. Thereafter, we perform a population-weighted average of the grids that lie within the political boundaries of the countries considered in this study. Gridded population data was obtained for 2015 from SEDAC (https://sedac.ciesin.columbia.edu/data/collection/gpw-v4).

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

SC, SKS, MJ, and PD designed the research. TK, SS, SC, SKS, and PD performed the research. TK, SS, SC, SKS, MJ, and PD analyzed the data. TK, SC, SKS, MJ, and PD wrote the paper. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

SS acknowledges the financial support from the Department of Science & Technology (DST), Government of India, under the scheme DST-Inspire (Grant No: IF160459). PD acknowledges financial support from the Science & Engineering Research Board (SERB), Government of India (Grant No: CRG/2019/002402). This manuscript has been released as a pre-print at medRxiv (49).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2020.569669/full#supplementary-material

REFERENCES

1. World Health Organization. *Coronavirus Disease 2019 (covid-19): Situation Report, 59*. Technical report, World Health Organization (2020).
2. Eurosurveillance Editorial Team. Note from the editors: World health organization declares novel coronavirus (2019-ncov) sixth public health emergency of international concern. *Eurosurveillance*. (2020) 25:200131e. doi: 10.2807/1560-7917.ES.2020.25.5.200131e
3. *Covid-19 Coronavirus Pandemic*. (2020). Available online at: https://www.worldometers.info/coronavirus/
4. Wu JT, Leung K, Bushman M, Kishore N, Niehus R, de Salazar PM, et al. Estimating clinical severity of covid-19 from the transmission dynamics in Wuhan, China. *Nat Med*. (2020) 26:506–10. doi: 10.1038/s41591-020-0822-7
5. Kraemer MUG, Yang C-H, Gutierrez B, Wu C-H, Klein B, Pigott DM, et al. The effect of human mobility and control measures on the covid-19 epidemic in China. *Science*. (2020) 368:493–7. doi: 10.1126/science.abb4218
6. Singh R, Adhikari R. Age-structured impact of social distancing on the covid-19 epidemic in india. *arXiv*. (2020) arXiv:2003.12055.
7. Mahase E. Covid-19: UK starts social distancing after new model points to 260,000 potential deaths. *Science*. (2020) 368:m1089. doi: 10.1136/bmj.m1089
8. *Coronavirus in New York: Lunar New Year Events Canceled Over Fears*. (2020). Available online at: https://www.nytimes.com/2020/01/29/nyregion/coronavirus-nyc.html
9. Hatchett RJ, Mecher CE, Lipsitch M. Public health interventions and epidemic intensity during the 1918 influenza pandemic. *Proc Natl Acad Sci USA*. (2007) 104:7582–7. doi: 10.1073/pnas.0610941104

10. Bootsma MCJ, Ferguson NM. The effect of public health measures on the 1918 influenza pandemic in us cities. *Proc Natl Acad Sci USA*. (2007) 104:7588–93. doi: 10.1073/pnas.0611071104
11. Ferguson N, Laydon D, Gilani GN, Imai N, Ainslie K, Baguelin M, et al. *Report 9: Impact of Non-pharmaceutical Interventions (NPIS) to Reduce Covid19 Mortality and Healthcare Demand*. Technical report, Imperial College London (2020).
12. Anderson RM, Heesterbeek H, Klinkenberg D, Hollingsworth TD. How will country-based mitigation measures influence the course of the covid-19 epidemic? *Lancet*. (2020) 395:931–4. doi: 10.1016/S0140-6736(20)30567-5
13. Prem K, Liu Y, Russell TW, Kucharski AJ, Eggo RM, Davies N, et al. The effect of control strategies to reduce social mixing on outcomes of the covid-19 epidemic in Wuhan, China: a modelling study. *Lancet Public Health*. (2020) 5:e261–70. doi: 10.1016/S2468-2667(20)30073-6
14. O'Regan SM, Drake JM. Theory of early warning signals of disease emergence and leading indicators of elimination. *Theor Ecol*. (2013) 6:333–57. doi: 10.1007/s12080-013-0185-5
15. Brett TS, Drake JM, Rohani P. Anticipating the emergence of infectious diseases. *J R Soc Interface*. (2017) 14:20170115. doi: 10.1098/rsif.2017.0115
16. O'Dea EB, Drake JM. Disentangling reporting and disease transmission. *Theor Ecol*. (2019) 12:89–98. doi: 10.1007/s12080-018-0390-3
17. Drake JM, Brett TS, Chen S, Epureanu BI, Ferrari MJ, Marty É, et al. The statistics of epidemic transitions. *PLoS Comput Biol*. (2019) 15:e1006917. doi: 10.1371/journal.pcbi.1006917
18. Harris MJ, Hay SI, Drake JM. Early warning signals of malaria resurgence in Kericho, Kenya. *Biol Lett*. (2020) 16:20190713. doi: 10.1098/rsbl.2019.0713
19. Kefi S, Dakos V, Scheffer M, Van Nes EH, Rietkerk M. Early warning signals also precede non-catastrophic transitions. *Oikos*. (2013) 122:641–8. doi: 10.1111/j.1600-0706.2012.20838.x
20. Liu Y, Gayle AA, Wilder-Smith A, Rocklv J. The reproductive number of COVID-19 is higher compared to SARS coronavirus. *J Travel Med*. (2020) 27:taaa021. doi: 10.1093/jtm/taaa021
21. Dibble CJ, O'Dea EB, Park AW, Drake JM. Waiting time to infectious disease emergence. *J R Soc Interface*. (2016) 13:20160540. doi: 10.1098/rsif.2016.0540
22. Scheffer M, Carpenter SR, Lenton TM, Bascompte J, Brock WA, Dakos V, et al. Anticipating critical transitions. *Science*. (2012) 338:344–8. doi: 10.1126/science.1225244
23. Dakos V, Carpenter SR, Brock WA, Ellison AM, Guttal V, Ives AR, et al. Methods for detecting early warnings of critical transitions in time series illustrated using simulated ecological data. *PLoS ONE*. (2012) 7:e41010. doi: 10.1371/journal.pone.0041010
24. Wang R, Dearing JA, Langdon PG, Zhang E, Yang X, Dakos V, et al. Flickering gives early warning signals of a critical transition to a eutrophic lake state. *Nature*. (2012) 492:419. doi: 10.1038/nature11655
25. Scheffer M, Bascompte J, Brock WA, Brovkin V, Carpenter SR, Dakos V, et al. Early-warning signals for critical transitions. *Nature*. (2009) 461:53–9. doi: 10.1038/nature08227
26. Sarkar S, Sinha SK, Levine H, Jolly MK, Dutta PS. Anticipating critical transitions in epithelial-hybrid-mesenchymal cell-fate determination. *Proc Natl Acad Sci USA*. (2019) 116:26343–52. doi: 10.1073/pnas.1913773116
27. Dakos V, Scheffer M, van Nes EH, Brovkin V, Petoukhov V, Held H. Slowing down as an early warning signal for abrupt climate change. *Proc Natl Acad Sci USA*. (2008) 105:14308–12. doi: 10.1073/pnas.0802430105
28. Scheffer M. *Critical Transitions in Nature and Society*. Princeton, NJ: Princeton University Press (2009).
29. May RM, Levin SA, Sugihara G. Complex systems: ecology for bankers. *Nature*. (2008) 451:893–5. doi: 10.1038/451893a
30. Scheffer M, Carpenter S, Foley JA, Folke C, Walker B. Catastrophic shifts in ecosystems. *Nature*. (2001) 413:591. doi: 10.1038/35098000
31. Scheffer M, Bolhuis JE, Borsboom D, Buchman TG, Gijzel SMW, Goulson D, et al. Quantifying resilience of humans and other animals. *Proc Natl Acad Sci USA*. (2018) 115:11883–90. doi: 10.1073/pnas.1810630115
32. Dakos V, Van Nes EH, d'Odorico P, Scheffer M. Robustness of variance and autocorrelation as indicators of critical slowing down. *Ecology*. (2012) 93:264–71. doi: 10.1890/11-0889.1
33. Dutta PS, Sharma Y, Abbott KC. Robustness of early warning signals for catastrophic and non-catastrophic transitions. *Oikos*. (2018) 127:1251–63. doi: 10.1111/oik.05172
34. Brett T, Ajelli M, Liu Q-H, Krauland MG, Grefenstette JJ, van Panhuis WG, et al. Detecting critical slowing down in high-dimensional epidemiological systems. *PLoS Comput Biol*. (2020) 16:e1007679. doi: 10.1371/journal.pcbi.1007679
35. Ribeiro SP, Dattilo W, Castro e Silva A, Reis AB, Goes-Neto A, Alcantara L, et al. Severe airport sanitarian control could slow down the spreading of covid-19 pandemics in brazil. *medRxiv*. (2020).
36. Matamalas JT, Gómez S, Arenas A. Abrupt phase transition of epidemic spreading in simplicial complexes. *Phys Rev Res*. (2020) 2:012049. doi: 10.1103/PhysRevResearch.2.012049
37. Linton NM, Kobayashi T, Yang Y, Hayashi K, Akhmetzhanov AR, Jung S, et al. Incubation period and other epidemiological characteristics of 2019 novel coronavirus infections with right truncation: a statistical analysis of publicly available case data. *J Clin Med*. (2020) 9:538. doi: 10.3390/jcm9020538
38. Li R, Pei S, Chen B, Song Y, Zhang T, Yang W, et al. Substantial undocumented infection facilitates the rapid dissemination of novel coronavirus (SARS-CoV2). *Science*. (2020) 368:489–93. doi: 10.1126/science.abb3221
39. Venter ZS, Aunan K, Chowdhury S, Lelieveld J. Covid-19 lockdowns cause global air pollution declines. *Proc Natl Acad Sci USA*. (2020). 117:18984–90. doi: 10.1073/pnas.2006853117
40. Jacob DJ, Winner DA. Effect of climate change on air quality. *Atmos Environ*. (2009) 43:51–63. doi: 10.1016/j.atmosenv.2008.09.051
41. Gillespie DT. Stochastic simulation of chemical kinetics. *Annu Rev Phys Chem*. (2007) 58:35–55. doi: 10.1146/annurev.physchem.58.032806.104637
42. Guest JL, del Rio C, Sanchez T. The 3 steps needed to end the covid-19 pandemic: Bold public health leadership, rapid innovations, and courageous political will. *JMIR Public Health Surveill*. (2020) 6:e19043. doi: 10.2196/19043
43. *Coronavirus Disease 2019 (Covid-19) Situation Report 72*. (2020). Available online at: https://www.who.int/docs/default-source/coronaviruse/situation-reports/20200401-sitrep-72-covid-19.pdf?sfvrsn=3dd8971b_2
44. *Coronavirus Disease 2019 (Covid-19) Situation Report 74*. (2020). Available online at: https://www.who.int/docs/default-source/coronaviruse/situation-reports/20200403-sitrep-74-covid-19-mp.pdf?sfvrsn=4e043d03_14
45. *Coronavirus Disease 2019 (Covid-19) Situation Report 76*. (2020). Available online at: https://www.who.int/docs/default-source/coronaviruse/situation-reports/20200405-sitrep-76-covid-19.pdf?sfvrsn=6ecf0977_2
46. Krotkov NA, Lamsal LN, Celarier EA, Swartz WH, Marchenko SV, Bucsela EJ, et al. The version 3 OMI NO_2 standard product. *Atmos Meas Tech*. (2017) 10:3133–49. doi: 10.5194/amt-10-3133-2017
47. Wang Y, Beirle S, Lampel J, Koukouli M, De Smedt I, Theys N, et al. Validation of OMI. GOME-2A and GOME-2B tropospheric NO_2, SO_2 and HCHO products using MAX-DOAS observations from 2011 to 2014 in Wuxi, China: investigation of the effects of priori profiles and aerosols on the satellite products. *Atmos Chem Phys*. (2017) 17:5007–33. doi: 10.5194/acp-17-5007-2017
48. Lamsal LN, Krotkov NA, Celarier EA, Swartz WH, Pickering KE, Bucsela EJ, et al. Evaluation of OMI operational standard NO_2 column retrievals using in situ and surface-based NO_2 observations. *Atmos Chem Phys*. (2014) 14:11587. doi: 10.5194/acp-14-11587-2014
49. Kaur T, Sarkar S, Chowdhury S, Sinha SK, Jolly MK, Dutta PS. Anticipating the novel coronavirus disease (covid-19) pandemic. *medRxiv*. (2020).

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Medicine

OPINION
published: 03 September 2020
doi: 10.3389/fmed.2020.00486

A Citizen Science Facemask Experiment and Educational Modules to Improve Coronavirus Safety in Communities and Schools

*Sarah E. Eichler[1], Austin P. Hopperton[2], Juan José Alava[3], Antonio Pereira Jr.[4], Rukhsana Ahmed[5], Zisis Kozlakidis[6], Sanja Ilic[7] and Alexander Rodriguez-Palacios[2,8]**

[1] Department of Biological Sciences, Kent State University at Salem, Salem, OH, United States, [2] Division of Gastroenterology and Liver Disease, School of Medicine, Case Western Reserve University, Cleveland, OH, United States, [3] Institute for Oceans and Fisheries, University of British Columbia, Vancouver, BC, Canada, [4] Institute of Technology, Federal University of Pará, Belém, Brazil, [5] Department of Communication, University at Albany, SUNY, Albany, NY, United States, [6] International Agency for Research on Cancer, World Health Organization, Lyon, France, [7] Department of Human Sciences, The Ohio State University, Columbus, OH, United States, [8] University Hospitals Research and Education Institute, University Hospitals Cleveland Medical Center, Cleveland, OH, United States

Keywords: COVID-19, mass media, health communication, prevention, intervention, social behavioral changes, facemask, school education

OPEN ACCESS

Edited by:
Longxiang Su,
Peking Union Medical College Hospital (CAMS), China

Reviewed by:
Holmes Finch,
Ball State University, United States
Matjaž Perc,
University of Maribor, Slovenia

***Correspondence:**
Alexander Rodriguez-Palacios
axr503@case.edu

Specialty section:
This article was submitted to Infectious Diseases-Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: *22 May 2020*
Accepted: *17 July 2020*
Published: *03 September 2020*

Citation:
Eichler SE, Hopperton AP, Alava JJ, Pereira A Jr, Ahmed R, Kozlakidis Z, Ilic S and Rodriguez-Palacios A (2020) A Citizen Science Facemask Experiment and Educational Modules to Improve Coronavirus Safety in Communities and Schools. Front. Med. 7:486. doi: 10.3389/fmed.2020.00486

INTRODUCTION

According to the UNICEF, children between 0–14 years represent ∼26% of the total global population (∼45% in Africa; 22% in USA, of which 90% attend school; ranging from 85–100% across countries) (1). With high case-fatality ratios between 4.5–7.5% (Germany/Iran/USA/Brazil/Canada) and 11.9–16.4% (Spain/Italy/UK/France/Belgium) (2), there is a critical need to empower citizens, especially children (often asymptomatic carriers), with education strategies to control COVID-19. Especially, there is need to support facemask citizen science and experiential education among children and families as the globe exits from the current lockdown, and teachers and students desire and seek for safe strategies to return to densely-attended schools. COVID-19 is a pandemic respiratory disease that disseminates as infectious respiratory or saliva droplets are released into the environment as people talk, sneeze, and cough (3–5). Currently the most publicized methods to prevent local transmission of COVID-19 and promote "droplet safety" in hospitals and communities include hand washing, social distancing, and stay-at-home strategies. In contrast to established benefits for medical masks in hospitals, the benefits of wearing masks or face covers/coverings (hereafter, "facemask") in the community have been inconsistently debated by the media, creating confusion, and misinformation (6). Furthermore, high-profile political leaders in countries heavily affected by the pandemic have given misleading signs regarding containment measures associated with COVID-19 (7–11) increasingly polarizing local communities around arguments on the value of facemasks in promoting public health, which is critically important to incentivize during the emergence of citizens from their lockdowns and during the phase of reopening local economies.

DROPLET AND FACEMASK SCIENCE TO EMPOWER COMMUNITY ACTION

Two of the most important measures recommended for public areas are social distancing and wearing facemasks to prevent COVID-19. Of these, facemasks predetermines the minimum person-person distance required in social distancing. That is, facemask utilization allows for safer interactions at closer distances because droplets are contained within the mask. However,

such concept is not well publicized and facemasks value have received contradictory attention. Inconsistent compliance with droplet control strategies now threatens COVID-19 containment. Governments that led initiatives against COVID-19 have reversed orders on facemasks as "mandatory" due to protests, highlighting that cultural acceptability is necessary for the success of any measure intended to control COVID-19. The science of facemasks is straightforward (12), facemasks stop (97.2–99.7%) droplets dispersion (12–15). Thus, the reversal of mask-wearing mandates leaves education and self-awareness as our ultimate strategies to ensure equitable and sustained public droplet safety.

Adapting a recent spray-bottle "sneeze-simulation" technique (14), we propose an educational module to promote citizen science for greater understanding of facemask effectiveness to protect the environment and the general public. Completion of simple home or school-based activities followed by online, volunteered data submission, with graphical feedback summaries encourages understanding, and quantitative learning about facemasks. Our module will help improve the knowledge about germ dissemination by droplets, which is less confusing than teaching the abstract concept of prevention of disease for the wearer. This citizen science educational campaign could inspire the sustained routine wearing of facemasks and solidarity in the community, leading to COVID-19 reduction. Ubiquitous use of facemasks combined with international and multi-disciplinary cooperation at a global scale are necessary to overcome the pandemic (16) and also to prevent further infections and overburdened healthcare systems as recently forecasted (17).

COVID-19 COMMUNICATION AND FUTURE EDUCATION

Promoting established and new approaches to protect human health, effective public messaging, and health education programs are paramount during infectious disease outbreaks (18). This is especially true in the era of COVID-19 so that a second global wave of coronavirus infections does not surpass the first wave. COVID-19 has created many challenges and fears for society because of its high infection rate, rapid community transmission, and high mortality rate, in particular for the elderly and those with underlying health conditions. There are various health challenges across urban/rural gradients that may impact the future local rates of associated mortality. These include sanitation infrastructure, hygiene and health education, implementation of prevention measures, and variable access to health care relevant to COVID-19 diagnosis, monitoring, and treatment.

Moving forward, public health approaches need to remain nimble to allow improvement in interventions and research tools quickly enough to stay ahead of the pandemic trajectory. New collaborative efforts are vital to ensure that the health needs in various communities can be met (19, 20). One such approach is appropriate access to health education programs (21), and evidence-based solutions such as home-made, double-layered facemasks (22). Thus, communities are empowered equitably and sustainably to improve health outcomes through proactive citizen science and education (23). Increasing community understanding of personal and community prevention measures and widespread signage promoting droplet education and facemask use (24) could alleviate personal conflicts surrounding citizen-citizen requests to wear facemasks in public. Well-communicated and unified educational campaigns are important in primary/high-school and higher education institutions, where the majority of individuals are eager to return (to often highly-populated classrooms), and because facemask compliance and social distancing within most young communities are suboptimal for various reasons. Returning to schools without clarifying with students the science of droplets and facemasks will facilitate the spread or misinformation and conceptual polarization. With ∼26% of the population attending schools, education leaders have the great opportunity and potential to authoritatively help improve our collective knowledge and understanding of how and why it is important to increase the coronavirus safety in their local communities.

CONTRADICTING GUIDELINES ON FACEMASKS

Ongoing debates about the usefulness of facemasks for the public developed in response to contradicting global health directives widely-publicized early in the COVID-19 emergency (6, 25–30). International health and political leaders insisted that mask use by non-healthcare workers would not protect the public, but rather would exacerbate supply shortages needed for hospitals (31, 32). This logic was reiterated in numerous regions, including Australia, where similar "anti-masking" arguments featured prominently (33).

Reportedly, healthcare workers experienced high rates of infections and mortality, partly because insufficient access to personal protection equipment (PPE) increased droplet exposure risk (34, 35). As PPE shortages worsened, governments, and institutions resorted to public requests for PPE (e.g., using social media #GetMePPE) (36). Latin America has also experienced widespread PPE shortages, e.g., Ecuador was assisted by the Pan-American Health Organization with PPE in April 2020 (37).

Being increasingly aware of foreseen shortages, hospital systems made calls for donations of homemade facemasks for use by caregivers (38–42). As part of pandemic containment strategies, more than 50 countries outside the US have mandatory facemask policies (43). In those countries, there is apparent cultural acceptance and adoption of facemask guidelines; however, while the precautionary principle applies, more research on implementation measures is needed (44). As of May 11, 2020 several states removed their requirements for facemasks in public (45).

Further challenges recently emerged as local violence, conflict, and publicized protests attracted global public attention leading to some high-profile public officials reverting orders or declining to wear facemasks in public (46). Some of the reasons cited were political in origin (e.g., US civil rights movement legacy, and fears of increased racial profiling), and some reflected a lack of information regarding "what a facemask will or will not do" in reducing COVID-19. The lack of clear information,

combined with politicization in certain regions, could lead to medical advice being discounted–even though most people consider the use of facemasks protective (47, 48), which is in agreement with data showing reduction of transmission. Clear guidance and the dissemination of factual information in the community is needed as part of citizen science and education campaigns. Contributing to such educational strategies to inform and incentivize the public, our laboratory recently determined that textiles, when used as 2-layer-covers, can reduce the contamination of the environment with bacteria-carrying droplets by 99.7%, which also fully protects germ-free mice when their cages are covered with 2-layer textiles (13, 14, 49). To share this important finding with the public, we created an educational module on the effectiveness of facemasks, described in brief below and freely accessible in English, French, Spanish, and Portuguese at https://sites.google.com/kent.edu/face-mask-challenge/face-mask-challenge-home, which address many regions facing severe rates of COVID19 infection.

DROPLET SCIENCE AND DOOR SIGN EDUCATION

Telling people that they did not need masks early in the pandemic created public distrust, because official messages assumed that the public was unable to process facts, specifically, that masks indeed protect health-workers (6). To regain public confidence, we propose that there is a need to provide detailed information to the public regarding the basics of droplet science. The physics of droplet dynamics in disease dispersion has been studied for decades in agriculture and plant sciences as a means to understand disease control (50, 51). In medicine, there is increasing interest in the dynamics of human droplet production and contamination of the environment (4, 5, 49, 52–56).

Except for health, food, and some industry workers, it is sensible to assume that most people never used facemasks before the pandemic. Thus, droplet education campaigns to prevent respiratory pandemics should acknowledge the novelty of facemask interventions so that knowledge discovery via experimentation translates into greater compliance. Communities need instruction that masks should be worn effectively, and basic droplet science recognized.

Clinical observations have shown that masks do not always prevent disease in people at high risk, especially, because subjects often (∼50%) do not use masks properly, or all the time, and because ancillary measures like "handwashing" and "not touching their face" are often lax (57, 58). Prevention failures can be corrected with educational campaigns aimed at a broad range of public but especially school children provided that instructions emphasize practice. And practice can be achieved through hands-on activities.

Viruses live and replicate in liquid phases inside cells and as such can only be expelled in liquid droplets as mucus/saliva in company with other more abundant microorganisms that also live in the respiratory/oral system, including other viruses, bacteria, and fungi. To correct such source of confusion among citizens, herein we provide a graphical summary of the biology and physics of droplets in humans as they speak, cough, and sneeze (**Figure 1A**). Also we provide customizable door sign examples to promote public droplet safety amidst COVID-19 to promote widespread awareness while decreasing the need for citizen-citizen verbal interactions, reducing conflict risk [see resource in (24)]. By simulating sneeze droplet dynamics using recently validated rapid spray-bottle methods and inocuous bacterial suspensions (14), we also illustrate that mask wearing reduces the range of droplet dispersion, increasing the margin of droplet safety within person-person interaction distances (**Figures 1B–F**).

CITIZEN SCIENCE: DROPLET AND FACEMASK EXPERIMENTS FOR COMMUNITIES AND SCHOOLS

A community-based approach to enhance public health preparedness was fundamental during past emergencies with Hurricane Katrina (2005), and the H5N1 avian influenza (2004) and H1N1 pandemic (2009) (61). Local community preparedness during emergencies is recognized as an important goal but remains challenging to implement and monitor (62–64). During global emergencies, integrated approaches to delivering community health services can significantly improve the emergency management by creating supportive social contexts within which communities can withstand and recover from public health emergencies (65, 66). Appropriate communication of health response actions -or lack thereof- can impact both epidemiologic and economic trends (67). Integration of equity, health literacy (including information, scientific, digital, and numeracy literacies) (68), cultural tailoring (69), and educational efforts must all be considered to provide clear instruction and improved understanding of the benefits of facemask use in public. In our view, a major educational campaign is needed in order to build community cooperation toward wide scale use of face coverings to increase community resilience to both current and future infectious disease outbreaks such as COVID-19.

As a response to this call, an educational exercise and citizen science project is presented here to promote discussion and greater awareness of facemask effectiveness by providing an innovative hands-on activity. Based on a validated spray simulation method (13, 14), community members can observe first-hand how textile barriers stop the dispersal of colored droplets, made with coffee for instance, or stop the dispersal of germs using solutions containing microbes safely present in foods like yogurt or garden soil. The educational module contains four experiments of varying difficulty and is freely available as an archive of latest versions and community contributions (https://github.com/axr503/education), as a website in multiple languages (English, Spanish, French and Portuguese, specifically developed for this project, or amenable for automatic translation via online search engines such as Google Chrome, see also Figure 1G-H for direct smartphone access) at https://sites.google.com/kent.edu/face-mask-challenge/face-mask-challenge-home or as 'ready-to-print' PDF module files for direct public access in four languages (see **Supplementary Materials**).

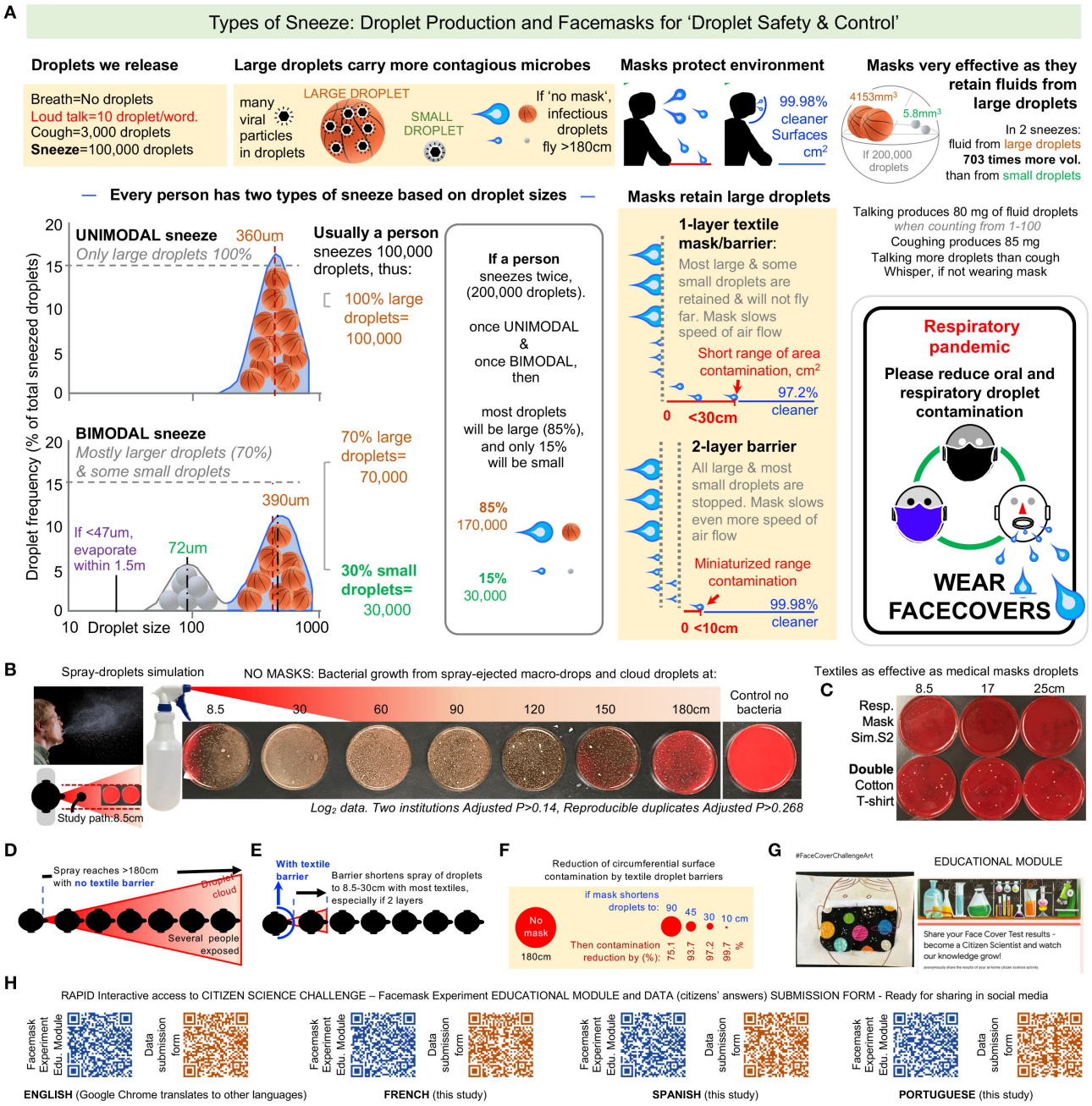


FIGURE 1 | Detailed educational citizen science modules could be based on information on droplet biology and experiments to increase experiential knowledge, facemask compliance, and public droplet safety. **(A)** Human sneezes produce the most droplets that are spread farther than 6 ft (180 cm) in which most are large droplets that contain far more viral particles than small droplets. Sneezes result in large droplets followed by a mix of large and small droplets in sizes proportional to a basketball relative to a golf ball, respectively. Virtually all large droplets and most small droplets are contained by a two-layer textile barrier, which reduces the resulting area of contamination (14, 55, 59, 60). Communicating the importance of face covers via door signs can reduce person-to-person verbal interactions and lessen exposure risk (see https://figshare.com/articles/Door_Signs_to_Promote_Public_Droplet_Safety_Amidst_COVID-19/12202808). **(B)** Infectious droplets can easily travel 180 cm (the recommended social distancing space) without a barrier as evidenced by bacterial colony forming units on agar plates in the line of culture spray, whereas **(C)** a loose double-layered t-shirt barrier reduced transmission of infectious particles nearly as thoroughly as a medical mask (49) **(D–F)**. A face cover results in 99.98% cleaner environmental surface following two sneezes- protecting both the wearer and others who may contact surfaces. **(G)** An example of a citizen science facemask experiment project can be accessed at **Supplementary Material** and the data report form at https://docs.google.com/forms/d/e/1FAIpQLSd9cV7HQzxr49MsC-icHCzxlOnlhX2z7e7iza3cJ-NGzJaFRw/viewform. **(H)** To rapidly enable the visualization of the module on mobile smart phones, reader should activate their cameras, point the camera to the QR-code, and then wait a couple of seconds for the phone to direct them to the website where the module or the answer submission forms are hosted. Studies supporting the droplet size and types of sneeze concepts in this graphical summary were adapted from (55, 59, 60). **(C–E)**, Unmodified from Rodriguez-Palacios et al. (14) under open CC BY license http://creativecommons.org/licenses/by/4.0/.

The online data submission form where the citizen scientists (adults, supervised children, or teachers) can share the results of their experiments with the global community, and see the aggregate responses globally or for their respective languages, is available at https://bit.ly/facemaskchallengedata.

This project is inspired by recent spray-simulation research that shows the effectiveness of cloth covers in reducing the contamination of the environment by liquid droplets (13, 14). Facemasks drastically cut environmental contamination to <8 cm (>99.7% reduction, if two-layers are used) compared to a radial area of contamination of 2 m if we do not wear droplet barriers (13, 14). Our module also helps demonstrate how many droplets people release into the environment during normal speech.

EDUCATIONAL MODULE CONTAINS FOUR EXPERIMENTS AVAILABLE IN MULTIPLE LANGUAGES

To enable a more uniform implementation of such education activities, the experiments are provided translated in four languages. Additional translations will also be possible. The educational module contains an introduction for teachers or parents and simple instructions for each of four home experiments that adults and supervised children could choose to complete. The activities are suitable for "citizen scientists" with at least a third-grade reading comprehension. In the activity, participants record the distance of droplet travel without any covering and compare dispersal patterns to those of a simulated sneeze that has been covered with a cloth. These citizen scientists can further investigate the spread of germs with and without a face cover by using home-made gelatin microbial growth plates to intercept and visualize germs spread through simulated sneeze or speech. Participants can choose to share their observations through an anonymous online (IRB-approved) form. Volunteered responses from the "#FaceMaskChallenge" will build a citizen science database and display simple graphs that illustrate the reduction in droplet spread.

The citizen science activity module is presented without political motivation or emotional language and adheres to basic scientific methods approach to knowledge discovery. Through the completion of a few hands-on activities, participants will discover details related to the effectiveness of facemasks including affordable and accessible home-made face covers. Questions and talking points in the module will encourage participants to think about community spread germs and their own choices regarding masks. Engagement with the science of public droplet safety may help move beyond the sensationalized discourse currently dominating the media and contribute to pandemic preparedness actions. This citizen science project with experiential educational modules (entitled - Facemask Challenge - A COVID-19 Educational Campaign To Promote Public Droplet Safety) is critically important to promote coronavirus safety. The educational campaign presented here and other experiential learning will support plans that ensure a safe return of students to classrooms, to protect children, prevent the asymptomatic dispersion of the virus, and protect vulnerable teachers, many of whom in the US school system (∼30%) are 50 years or older (70).

CONCLUSION

Scientific discoveries are often communicated to the public with uncertainty, unintentionally creating confusion among non-scientists. To control COVID-19, social distancing alone, as widely promoted (e.g., in radio stations, without encouraging facemasks), will be impossible to sustain as communities exit lockdowns. In the communication process, it is therefore important to realize that empowering citizens with tools to numerically visualize the benefits of a healthy behavior could facilitate the sustained experiential-based adoption of facemasks. The use of citizen science modules, as herein proposed, could improve cultural acceptability, community resilience and facilitate the development of educational strategies and policies to promote public droplet safety to control COVID-19. A straightforward "Test-your-Facemask" challenge and droplet experiment modules are herein released as a paper-and-online open-access resource for the benefit of the community. These resources can promote coronavirus safety in schools -protecting vulnerable populations, including experienced teachers, and families in local school districts as institutions seek strategies for a safe return to classrooms this year.

AUTHOR CONTRIBUTIONS

SE and AR-P developed the opinion concept. SE provided an initial draft and edited all versions. AR-P provided figure and edited all drafts. SE, AH, and SI tested supplemental module procedure. All the authors contributed substantially to discussion, development of text, corrections, and edits to the supplemental educational module and implementation to necessary translations. SE, AH, and AR-P prepared Supplementary Material to which all authors contributed. Individual authors provided translation of the Facemask Challenge module for Spanish (JA), French (ZK), and Portuguese (AP).

ACKNOWLEDGMENTS

The authors thank Maddie Rosemark for illustrations, Jessica Velez for comments on the educational module, Maissa Zeghidi, Diana Maria Rodriguez and Diego Alejandro Pulido R for their comments and remarkable support with the translations. JA thanks the Nippon Foundation for providing research funding.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2020.00486/full#supplementary-material

Supplementary Material | A compiled PDF document of the facemask challenge educational activity modules amenable for schools in English, French, Spanish and Portuguese. The links to the websites can be accessed from **Figures 1G,H** using smartphone cameras and the QR-codes.

REFERENCES

1. UNICEF 2017. *State of the World's Children Report*. (2017). Available online at: https://data.unicef.org/resources/state-worlds-children-2017-statistical-tables/
2. Johns Hopkins University. *Mortality Analyses - Johns Hopkins Coronavirus Resource Center*. (2020) Available online at: https://coronavirus.jhu.edu/data/mortality (accessed May 20, 2020).
3. Galbadage T, Peterson BM, Gunasekera RS. Does COVID-19 spread through droplets alone? *Front Public Heal.* (2020) 8:163. doi: 10.3389/fpubh.2020.00163
4. Techet BESAH, Bourouiba JWMBL. Visualization of sneeze ejecta : steps of fluid fragmentation leading to respiratory droplets. *Exp Fluids.* (2016) 57:1–9. doi: 10.1007/s00348-015-2078-4
5. Bourouiba L, Dehandschoewercker E, Bush JWM. Violent expiratory events : on coughing and sneezing. *J Fluid Mech.* (2014) 745:537–63. doi: 10.1017/jfm.2014.88
6. Tufekci Z. *Why Telling People They Don't Need Masks Backfired*. New York City, NY: New York Times (2020).
7. Arizona, Florida, Texas are latest coronavirus epicenters. Washington Post (2020).
8. Coronavirus: *"I'm all for masks," says Trump in change of tone - BBC News*. BBC News (2020) Available online at: https://www.bbc.com/news/world-us-canada-53258792 (accessed July 2, 2020).
9. Latino USA : NPR. How Brazil became the epicenter Of COVID-19. *Natl Public Radio.* (2020). Transcript: https://www.npr.org/2020/06/26/883829334/how-brazil-became-the-epicenter-of-covid-19
10. Andreoni M. *Brazil's Coronavirus Skeptic Leader Gets Judicial Scolding for Breaking Mask Law*. New York Times. (2020) Available online at: https://www.nytimes.com/2020/06/23/world/americas/bolsonaro-brazil-mask.html (accessed July 2, 2020).
11. Bates BR. The (In) Appropriateness of the WAR Metaphor in Response to SARS-CoV-2 : A Rapid Analysis of Donald J. *Trump's Rhetoric. Front Commun.* (2020) 5:50. doi: 10.3389/fcomm.2020.00050
12. Howard J. Masks help stop the spread of coronavirus – the science is simple and I'm one of 100 experts urging governors to require public mask-wearing. *Conversat.* (2020). Available online at: https://theconversation.com/masks-help-stop-the-spread-of-coronavirus-the-science-is-simple-and-im-one (accessed May 20, 2020)
13. Konda A, Prakash A, Moss GA, Schmoldt M, Grant GD, Guha S. Aerosol filtration efficiency of common fabrics used in respiratory cloth masks. *ACS Nano*. (2020) 14:6339–47. doi: 10.1021/acsnano.0c04676
14. Rodriguez-Palacios A, Cominelli F, Basson AR, Pizarro TT, Ilic S. Textile masks and surface covers – A spray simulation method and a universal droplet reduction model' against COVID-19 respiratory pandemic. *Front Med.* (2020) 7:260. doi: 10.3389/fmed.2020.00260
15. Howard J, Huang A, Li Z, Tufekci Z, Zdimal V, Westhuizen H-M van der, et al. Face masks against COVID-19: an evidence review. *Preprints*. (2020). doi: 10.20944/preprints202004.0203.v3
16. Momtazmanesh S, Ochs HD, Uddin LQ, Perc M, Routes JM, Vieira DN, et al. All together to Fight COVID-19. *Am J Trop Med Hyg.* (2020) 102:1181–3. doi: 10.4269/ajtmh.20-0281
17. Perc M, Gorišek Miksić N, Slavinec M, StoŽer A. Forecasting COVID-19. *Front Phys*. (2020) 8:1–5. doi: 10.3389/fphy.2020.00127
18. Wilder-Smith A, Freedman DO. Isolation, quarantine, social distancing and community containment: pivotal role for old-style public health measures in the novel coronavirus (2019-nCoV) outbreak. *J Travel Med.* (2020) 27:taaa020. doi: 10.1093/jtm/taaa020
19. Xiang YT, Jin Y, Cheung T. Joint international collaboration to combat mental health challenges during the coronavirus disease 2019 pandemic. *JAMA Psychiatry*. (2020). doi: 10.1001/jamapsychiatry.2020.1057
20. Al-Shamsi HO, Alhazzani W, Alhuraiji A, Coomes EA, Chemaly RF, Almuhanna M, et al. A practical approach to the management of cancer patients during the novel coronavirus disease 2019 (COVID-19) pandemic: an international collaborative group. *Oncologist.* (2020) 25:e936–45. doi: 10.1634/theoncologist.2020-0213
21. Ng Y-M, Peggy PL. Coronavirus disease (COVID-19) prevention: virtual classroom education for hand hygiene. *Nurse Educ Pract.* (2020) 45:102782. doi: 10.1016/j.nepr.2020.102782
22. Cheng KK, Lam TH, Leung CC. Wearing face masks in the community during the COVID-19 pandemic: altruism and solidarity. *Lancet.* (2020) 2019:2019–20. doi: 10.1016/S0140-6736(20)30918-1
23. Cheng VCC, Wong S-C, Chuang VWM, So SYC, Chen JHK, Sridhar S, et al. The role of community-wide wearing of face mask for control of coronavirus disease 2019 (COVID-19) epidemic due to SARS-CoV-2. *J Infect.* (2020) 81:107–14. doi: 10.1016/j.jinf.2020.04.024
24. Rodriguez-Palacios A. *Door Signs to Promote Public Droplet Safety Amidst COVID-19*. (2020). Available online at: https://figshare.com/articles/Door_Signs_to_Promote_Public_Droplet_Safety_Amidst_COVID-19/12202808 (accessed May 20, 2020).
25. World Health Organization. *Advice on the Use of Masks in the Context of COVID-19: Interim Guidance-2*. (2020). doi: 10.15557/PiMR.2020.0005
26. U.S. Centers for Disease Control and Prevention. *CDC: Recommendation Regarding the Use of Cloth Face Coverings*. Atlanta, GA (2020) Available online at: https://www.cdc.gov/coronavirus/2019-ncov/prevent-getting-sick/cloth-face-cover.html (accessed May 16, 2020).
27. Cohen J. Not wearing masks to protect against coronavirus is a 'big mistake,' top Chinese scientist says. *Science*. (2020). doi: 10.1126/science.abb9368
28. Africa CDC. *COVID-19 Scientific and Public Health Policy*. Addis Ababa (2020).
29. Editor. *Coronavirus: UK to be "Guided by Scientists" on Face Masks*. BBC News (2020)
30. European Centre for Disease Prevention and Control. *Using face masks in the community Reducing COVID-19 transmission from potentially asymptomatic or pre-symptomatic people through the use of face masks*. Stockholm (2020).
31. U.S. Surgeon General. *@Surgeon_General : "Seriously People- STOP BUYING MASKS! They are NOT Effective in Preventing General Public From Catching #Coronavirus, but if Healthcare Providers Can't Get Them to Care for Sick Patients, it Puts Them and Our Communities at Risk!* https: *Tweet*. (2020) Available online at: https://twitter.com/surgeon_general/status/1233725785283932160 (accessed May 16, 2020).
32. Sonnemaker T. How you can help doctors and nurses get desperately needed face masks - Business Insider. *Bus Insid*. (2020). Available online at: https://www.businessinsider.com/how-you-can-help-donate-face-masks-doctors-nurses-coronavirus-2020-3 (accessed May 16, 2020).
33. Mahase E. Novel coronavirus: Australian GPs raise concerns about shortage of face masks. *BMJ*. (2020) 368:m477. doi: 10.1136/bmj.m477
34. Balmer C, Pollina E. Italy's Lombardy asks retired health workers to join coronavirus fight. *World Econ Forum*. (2020). Available online at: https://www.weforum.org/agenda/2020/03/italys-lombardy-etired-health-workers-coronavirus-covid19-pandemic (accessed May 16, 2020).
35. Bradsher K, Alderman L. *The World Needs Masks. China Makes Them, but Has Been Hoarding Them. - The New York Times*. New York Times (2020).
36. Ranney ML, Griffeth V, Jha AK. Critical supply shortages - the need for ventilators and personal protective equipment during the Covid-19 pandemic. *N Engl J Med.* (2020) 382:e41. doi: 10.1056/NEJMp2006141
37. PAHO. *OPS/OMS Ecuador - OPS/OMS Dona al Ecuador Equipos de Protección Personal Para Apoyar Los Esfuerzos en la Respuesta al COVID 19*. (2020). Available online at: https://www.paho.org/ecu/index.php?option=com_content&view=article&id=2330:ops-oms-dona-al-ecuador-equipos-de-proteccion-personal-para-apoyar-los-esfuerzos-en-la-respuesta-al-covid-19&Itemid=360 (accessed May 16, 2020).
38. University Hospitals. *Donations & Volunteer to Make Face Masks for University Hospitals*. (2020). Available online at: https://www.uhhospitals.org/healthcare-update/masks-and-donations#masks (accessed May 16, 2020).
39. University of Iowa Health. *UI Health Care Seeks Donations of Cloth Face Masks as Well as Continued Donations of Personal Protective Equipment | University of IOWA Hospitals & Clinics*. (2020) Available online at: https://uihc.org/news/ui-health-care-seeks-donations-cloth-face-masks-well-continued-donations-personal-protective (accessed May 16, 2020).

40. Concord Hospital. *Concord Hospital Seeks Donations of Urgently Needed Supplies*. (2020). Available online at: https://www.concordhospital.org/about-us/news-publications/2020/medical-supply-donations/ (accessed May 16, 2020).
41. Deaconess Health. *Deaconess - How to Make a Face Mask*. (2020). Available online at: https://www.deaconess.com/How-to-make-a-Face-Mask (accessed May 16, 2020).
42. Frey L. Help Akron Children's protect our patients and staff with DIY face masks:inside children's blog. *Akron Child Hosp News Events*. (2020). Available online at: https://inside.akronchildrens.org/2020/04/01/help-akron-childrens-protect-our-patients-and-staff-with-diy-face-masks/ (accessed May 16, 2020).
43. Editor -Al Jazeera. *Which Countries Have Made Wearing Face Masks Compulsory*? Doha: Al Jazeera News (2020)
44. Greenhalgh T, Schmid MB, Czypionka T, Bassler D, Gruer L. Face masks for the public during the covid-19 crisis. *BMJ*. (2020) 369:m1435. doi: 10.1136/bmj.m1435
45. Conner K. *Face Masks in Cars, Stores, Outside: Places You're Expected to Wear a Covering*. San Francisco, CA: CNET (2020). Available online at: https://www.cnet.com/health/face-masks-in-cars-stores-outside-places-youre-expected-to-wear-a-covering/ (accessed May 16, 2020).
46. Letters to Editor- NYT. *Opinion: Maskless Mike Pence*. New York Times (2020).
47. Agüero F, Adell MN, Pérez Giménez A, López Medina MJ, Garcia Continente X. Adoption of preventive measures during and after the 2009 influenza A (H1N1) virus pandemic peak in Spain. *Prev Med.* (2011) 53:203–6. doi: 10.1016/j.ypmed.2011.06.018
48. Van D, McLaws ML, Crimmins J, MacIntyre CR, Seale H. University life and pandemic influenza: attitudes and intended behaviour of staff and students towards pandemic (H1N1) 2009. *BMC Public Health.* (2010) 10:130. doi: 10.1186/1471-2458-10-130
49. Rodriguez-Palacios A, Conger M, Cominelli F. Germ-free mice under two-layer textiles are fully protected from bacteria in sprayed microdroplets: a functional *in vivo* test of facemask-filtration materials. *Front. Med.* doi: 10.3389/fmed.2020.00504. [Epub ahead of print]
50. Nansen C, Ferguson JC, Moore J, Groves L, Emery R, Garel N, et al. Optimizing pesticide spray coverage using a novel web and smartphone tool, SnapCard. *Agron Sustain Dev.* (2015) 35:1075–85. doi: 10.1007/s13593-015-0309-y
51. Matthews GA. Elestrostatic spraying of pesticides: a review. *Crop Prot.* (1989) 8:3–15. doi: 10.1016/0261-2194(89)90093-8
52. Bourouiba L. A sneeze 1. *N Engl J Med.* (2016) 375:e15. doi: 10.1056/NEJMicm1501197
53. Chartier Y, Pessoa-Silva C. *Natural Ventilation for Infection Control in Health-Care Settings*. Bookshelf. World Health Organization (2009).
54. Kooij S, Sijs R, Denn MM, Villermaux E, Bonn D. What determines the drop size in sprays? *Phys Rev X.* (2018) 8:031019. doi: 10.1103/PhysRevX.8.031019
55. Han ZY, Weng WG, Huang QY. Characterizations of particle size distribution of the droplets exhaled by sneeze. *J R Soc Interface.* (2013) 10:20130560. doi: 10.1098/rsif.2013.0560
56. Burke W. Why do we sneeze? *Med Hypotheses.* (2012) 78:502–4. doi: 10.1016/j.mehy.2012.01.017
57. Chughtai AA, Seale H, MacIntyre CR. Use of cloth masks in the practice of infection control – evidence and policy gaps. *Int J Infect Control.* (2013) 9:1–12. doi: 10.3396/IJIC.v9i3.020.13
58. MacIntyre CR, Chughtai AA. Facemasks for the prevention of infection in healthcare and community settings. *BMJ.* (2015) 350:1–12. doi: 10.1136/bmj.h694
59. Xie X, Li Y, Sun H, Liu L. Exhaled droplets due to talking and coughing. *J R Soc Interface.* (2009) 6 (Suppl. 6):S703–14. doi: 10.1098/rsif.2009.0388.focus
60. Papineni RS, Rosenthal FS. The size distribution of droplets in the exhaled breath of healthy human subjects. *J Aerosol Med Depos Clear Eff Lung.* (1997) 10:105–16. doi: 10.1089/jam.1997.10.105
61. Plough A, Fielding JE, Chandra A, Williams M, Eisenman D, Wells KB, et al. Building community disaster resilience: perspectives from a large urban county department of public health. *Am J Public Health.* (2013) 103:1190–7. doi: 10.2105/AJPH.2013.301268
62. U.S. Centers for Disease Control and Prevention. *Public Health Preparedness Capabilities: National Standards for State and Local Planning, Updated*. (2019).
63. Cantey PT, Chuk MG, Kohl KS, Herrmann J, Weiss P, Graffunder CM, et al. Public Health Emergency Preparedness. *J Public Heal Manag Pract.* (2013) 19:70–6. doi: 10.1097/PHH.0b013e31824d4666
64. Biddinger PD, Savoia E, Massin-Short SB, Preston J, Stoto MA. Public health emergency preparedness exercises: Lessons learned. *Public Health Rep.* (2010) 125:100–6. doi: 10.1177/00333549101250S514
65. Groom AV, Jim C, LaRoque M, Mason C, McLaughlin J, Neel L, et al. Pandemic Influenza Preparedness and Vulnerable Populations in Tribal Communities. *Am J Public Health.* (2009) 99:S271–8. doi: 10.2105/AJPH.2008.157453
66. Breen MJ, Stuart AJ, Graham P. A community-based approach to influenza assessment and treatment during a pandemic. *Healthc Manag Forum.* (2007) 20:6–9. doi: 10.1016/S0840-4704(10)60250-5
67. Smith RD. Responding to global infectious disease outbreaks: lessons from SARS on the role of risk perception, communication and management. *Soc Sci Med.* (2006) 63:3113–23. doi: 10.1016/j.socscimed.2006.08.004
68. Hoffman-Goetz L, Donelle L, Ahmed R. *Health literacy in Canada: A Primer for Students*. Toronto, ON: Canadian Scholars' Press (2014).
69. Huang Y, Shen F. Effects of cultural tailoring on persuasion in cancer communication: a meta-analysis. *J Commun.* (2016) 66:694–715. doi: 10.1111/jcom.12243
70. (NCES). *National Center for Education Statistics & Schools and Staffing Survey. Public School Teacher Data File.* (2012). Available online at: https://nces.ed.gov/surveys/sass/

Disclaimer: Where authors are identified as personnel of the International Agency for Research on Cancer/WHO, the authors alone are responsible for the views expressed in this article and they do not necessarily represent the decisions, policy or views of the International Agency for Research on Cancer/WHO.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Medicine

ORIGINAL RESEARCH
published: 04 September 2020
doi: 10.3389/fmed.2020.552002

Clinical Characteristics and Outcomes of Severe and Critical Patients With 2019 Novel Coronavirus Disease (COVID-19) in Wenzhou: A Retrospective Study

Song-Zan Qian[1,2†], *Wan-dong Hong*[3†], *Lingjie-mao*[4], *Chenfeng-lin*[4], *Zhendong-fang*[4] *and Jing-Ye Pan*[1,2*]

[1] Department of Intensive Care Unit, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou, China, [2] Wenzhou Key Laboratory of Critical Care and Artificial Intelligence, Wenzhou, China, [3] Department of Gastroenterology, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou, China, [4] School of the First Clinical Medical Sciences, Wenzhou Medical University, Wenzhou, China

OPEN ACCESS

Edited by:
Ata Murat Kaynar,
University of Pittsburgh, United States

Reviewed by:
Mauricio Machado,
Faculty of Medicine of São José Do Rio Preto, Brazil
Andre M. Japiassu,
Oswaldo Cruz Institute, Oswaldo Cruz Foundation (Fiocruz), Brazil

***Correspondence:**
Jing-Ye Pan
wmupanjingye@126.com

[†] These authors have contributed equally to this work

Specialty section:
This article was submitted to Intensive Care Medicine and Anesthesiology, a section of the journal Frontiers in Medicine

Received: 15 April 2020
Accepted: 20 August 2020
Published: 04 September 2020

Citation:
Qian S-Z, Hong W-d, Lingjie-mao, Chenfeng-lin, Zhendong-fang and Pan J-Y (2020) Clinical Characteristics and Outcomes of Severe and Critical Patients With 2019 Novel Coronavirus Disease (COVID-19) in Wenzhou: A Retrospective Study. Front. Med. 7:552002. doi: 10.3389/fmed.2020.552002

Information about severe cases of 2019 novel coronavirus disease (COVID-19) infection is scarce. The aim of this study was to report the clinical characteristics and outcomes of severe and critical patients with confirmed COVID-19 infection in Wenzhou city. In this single-centered, retrospective cohort study, we consecutively enrolled 37 RT-PCR confirmed positive severe or critical patients from January 28 to February 16, 2020 in a tertiary hospital. Outcomes were followed up until 28-day mortality. Fifteen severe and 22 critical adult patients with the COVID-19 infection were included. Twenty-six (68.4%) were men. Echocardiography data results suggest that normal or increased cardiac output and diastolic dysfunction are the most common manifestations. Compared with severe patients, critical patients were older, more likely to exhibit low platelet counts and high blood urea nitrogen, and were in hospital for longer. Most patients had organ dysfunction during hospitalization, including 11 (29.7%) with ARDS, 8 (21.6%) with acute kidney injury, 17 (45.9%) with acute cardiac injury, and 33 (89.2%) with acute liver dysfunction. Eighteen (48.6%) patients were treated with high-flow ventilation, 9 (13.8%) with non-invasive ventilation, 10 (15.4%) with invasive mechanical ventilation, 7 (18.9%) with prone position ventilation, 6 (16.2%) with extracorporeal membrane oxygenation (ECMO), and 3 (8.1%) with renal replacement therapy. Only 1 (2.7%) patient had died in the 28-day follow up in our study. All patients had bilateral infiltrates on their chest CT scan. Twenty-one (32.3%) patients presented ground glass opacity (GGO) with critical patients more localized in the periphery and the center. The mortality of critical patients with the COVID-19 infection is low in our study. Cardiac function was enhanced in the early stage and less likely to develop into acute cardiac injury, but most patients suffered with acute liver injury. The CT imaging presentations of COVID-19 in critical patients were more likely with consolidation and bilateral lung involvement.

Keywords: COVID-19, infection, severity, critically ill, outcome

INTRODUCTION

Since December 2019, the outbreak of the novel coronavirus that originated in Wuhan has spread to more than 100 countries in Asia, Europe, North America, and the Middle East, and has become a global threat to human health. In February 2020, the World Health Organization designated the disease COVID-19 (1). More than 80,000 people have been infected in China, South Korea, Iran, and Italy, who are coping with significant outbreaks. Many studies have reported the clinical, epidemiological, laboratory, and radiological characteristics, and also treatment and clinical outcomes of patients confirmed with COVID-19 pneumonia (2–5). However, most of those studies focused mainly on Wuhan or Hubei. There are significant regional differences in the outcomes of COVID-19 in Wuhan and elsewhere. The pathophysiology of COVID-19 has gradually been recognized, the mortality of patients with COVID-19 in Wuhan was significantly higher than that in other regions. Understanding the clinical characteristics of patients in other regions outside Wuhan is really necessary for implementing different levels of prevention and control measures.

By February 17, 2020, there were 504 confirmed cases reported according to a government announcement in Wenzhou. Yang.et al presented the clinical characteristics and chest CT scan manifestations of most of the mild patients in Wenzhou, but did not report the severe COVID-19 characteristics (6). Aimed at exploring clinical characteristics and outcomes of hospitalized severe or critical patients with confirmed COVID-19 infection in Wenzhou, here, we show details of those patients admitted to the First Affiliated Hospital of Wenzhou Medical University and clinical outcome as of 28-day mortality.

MATERIALS AND METHODS

Study Design and Participants

For this single-centered, retrospective cohort study, we recruited adult inpatients (≥18 years old) admitted to The First Affiliated Hospital of Wenzhou Medical University, the only designated hospital in Wenzhou for patients with a severe or critical COVID-19 infection. The patients were consecutively enrolled from January 28 to February 16. Diagnosed with COVID-19 according to a laboratory reverse transcription polymerase chain reaction (RT-PCR) test, all these adult patients were confirmed as having the COVID-19 infection. The study was approved by The First Affiliated Hospital of Wenzhou Medical University Ethics Committee.

Data Collection

We retrospectively collected the medical records which included epidemiological, demographic, symptoms, laboratory results, complications, outcome, and treatment data. We collected the admission data of these patients. Laboratory confirmation of COVID-19 was done by real-time RT-PCR methods.

The data on age, sex, exposure history, comorbidity (hypertension, diabetes cardiovascular disease, chronic kidney disease, chronic liver disease, cerebrovascular disease, hematological diseases), symptoms from onset to hospital admission (fever, cough, expectoration, dyspnea, muscle pain, headache, sore throat, chill, diarrhea, fatigue), laboratory results on admission (hemoglobin, white blood cell count, neutrophil count, lymphocyte count, monocyte count, platelet, d-dimer, creatine kinase, creatine kinase–mb, alanine aminotransferase, aspartate aminotransferase, total bilirubin, blood urea nitrogen, creatinine, hypersensitive troponin I, procalcitonin, brain natriuretic peptide, lactate, albumin, total cholesterol, cytokine levels, T lymphocyte cell subsets), treatment [Glucocorticoid therapy, Immunoglobulin therapy, Thymosin, Thaliduan, Antibiotic treatment, Antiviral treatment, Oxygen Treatment, Prone Position Ventilation, Continuous renal replacement therapy (CRRT)], clinical outcome [Sepsis, Septic Shock, Acute respiratory distress syndrome (ARDS), Acute cardiac injury, Acute kidney injury, Acute liver injury, Secondary infection, Acidosis, Prognosis], and radiological and echocardiography data and as well as living status were collected. The Acute Physiology and Chronic Health Evaluation II (APACHEII) and SOFA score system were used to assess pneumonia severity.

Definitions

Sepsis and septic shock were diagnosed according to sepsis-3.0 definition (7). Acute kidney injury was defined according to the KDIGO clinical practice guidelines; acute cardiac injury was diagnosed if serum levels of cardiac biomarkers (e.g., high sensitive cardiac troponin I or brain natriuretic peptide) increased. Acute respiratory distress syndrome (ARDS) was defined by the Berlin definition. Acute liver injury was diagnosed if serum levels of liver biomarkers (e.g., Alanine aminotransferase, Aspartate aminotransferase, Total bilirubin) increased. Secondary infection was defined when patients had a positive culture of a new pathogen after admission. The disease severity (severe or critical) was defined according to the 6th edition guideline issued by China's National Health Commission (7).

Statistical Analysis

Continuous variables were presented as medians (IQR) or mean (SD) and compared with the Mann–Whitney *U*-test. Categorical variables were presented as *n* (%) and were compared by the chi-square or Fisher exact test between severe and critical patients with COVID-19. We used SPSS (version 22.0) for all analyses. A $p < 0.05$ was considered as statistically significant, statistical analyses were done using the SAS software, version 24.0.

RESULTS

By February 16, 2020, 37 severe or critical patients had been admitted to The First Affiliated Hospital of Wenzhou Medical University with confirmed COVID-19 pneumonia, of whom, 15 (40.5%) severe and 22 (59.5%) critical. The mean age was 57 years (21–93), 14 (21.5%) patients were older than 60 years old. Twenty-six (68.4%) patients were men. Ten (26.3%) patients recently visited Wuhan or Hubei, and 13 (34.2%) had contact with Wuhan residents, 4 (10.5%) had been exposed to a confirmed case, 11 (28.9%) patients had no definite causes. The most common chronic diseases were hypertension [14(36.8%)]

TABLE 1 | Baseline characteristics of patients infected with COVID-19.

Variable	ALL patients ($n = 37$)	Severe ($n = 15$)	Critically severe ($n = 22$)	p
Clinical parameters				
Age, median (IQR), year	55 (48–68)	54 (48–60)	56 (47–73)	0.132
>60 year	14 (21.5%)	3 (20.0%)	10 (45.5%)	0.111
Male gender, *n* (%)	26 (68.4%)	11 (73.3%)	15 (76.2%)	0.736
hospital-stay, mean (SD), d	30.4 (14.7)	25.1 (15.8)	31.9 (11.1)	0.134
Exposure history no. (%)				
Recently visited Wuhan or hubei	10 (26.3%)	4 (26.7%)	6 (27.3%)	0.967
Contact with Wuhan residents	13 (34.2%)	7 (46.7%)	6 (27.3%)	0.225
Exposure to patients*	4 (10.5%)	1 (6.7%)	3 (13.6%)	0.503
No definite causes	11 (28.9%)	4 (26.7%)	7 (31.9%)	0.875
Comorbidity no. (%)				
Hypertension	14 (36.8%)	6 (40.0%)	8 (36.4%)	0.823
Diabetes	8 (21.1%)	3 (20%)	5 (36.4%)	0.843
Cardiovascular disease	1 (2.6%)	0	1 (2.6%)	–
Chronic kidney disease	1 (2.6%)	0	1 (2.6%)	–
Chronic liver disease	1 (2.6%)	0	1 (4.5%)	–
Cerebrovascular disease	1 (2.6%)	0	1 (2.6%)	–
Hematological diseases	1 (2.6%)	0	1 (2.6%)	–
Signs and symptoms no. (%)				
Fever	37 (100%)	15 (100%)	22 (100%)	–
Cough	29 (78.4%)	11 (73.3%)	18 (81.8%)	0.835
Expectoration	16 (43.2%)	6 (40%)	10 (45.5%)	0.742
Dyspnea	8 (21.6%)	2 (13.3%)	6 (27.3%)	0.545
Muscle pain	4 (10.8%)	2 (13.3%)	2 (9.1%)	1.000
Headache	2 (5.4%)	2 (13.3%)	0 (0%)	0.158
Sore throat	4 (10.8%)	3 (20%)	1 (4.5%)	0.344
Chill	8 (21.6%)	5 (33.3%)	3 (13.6%)	0.307
Diarrhea	2 (5.4%)	1 (6.7%)	1 (4.5%)	1.000
Fatigue	7 (18.9%)	2 (13.3%)	5 (22.7%)	0.773
Scoring sytems				
APACHEII, median (IQR)	8 (4.5–10.5)	7 (4–9)	10 (6–13)	0.042
SOFA, median (IQR)	1 (0–3)	0 (0–2)	2 (1–3)	0.012

*APACHEII, The median Acute Physiology and Chronic Health Evaluation II; SOFA, sequential organ failure assessment; Data are n (%) or mean (SD) median (IQR); patients, *Patients who have confirmed COVID-19 infection or are highly suspected of being infected.*

and diabetes [8(21.1%)]. Twenty-two patients had increased serum levels of aspartate aminotransferase. All of the 37 patients had bilateral infiltrates on their chest CT scan. The most common symptoms were fever (100%), cough (78.4%), and expectoration (43.2%). The median APACHE II score and SOFA score of all patients were 8.0 (4.5–10.5) and 1 (0–3) (detail in **Table 1**).

Most patients had organ dysfunction during hospitalization, including 11 (29.7%) with ARDS, 8 (21.6%) with acute kidney injury, 17 (45.9%) with acute cardiac injury, and 33 (89.2%) with acute liver dysfunction. Only 3 patients (8.1%) had hypersensitive troponin I increased on admission. Eighteen (48.6%) patients were treated with high-flow ventilation, 9 (13.8%) with non-invasive ventilation, 10 (15.4%) with invasive mechanical ventilation, 7 (18.9%) with prone position ventilation, 6 (16.2%) with extracorporeal membrane oxygenation (ECMO), 3 (8.1%) with renal replacement therapy. 37 (100%) received antibacterial agents and antiviral treatment, 21 (56.8%) patients received glucocorticoids. Thymosin was treated in 21 (56.8%) of the patients. Thaliana was treated in 15 (40.5%) of the patients (Details in **Tables 2**, **3**).

Among the 37 severe or critical patients with the COVID-19 infection, only 1 (2.7%) patient died, after 14 days. Compared with severe patients, critical patients were older [52.4 (21–81) vs. 60.1 (39–93)], and had high APACHE II [7 (4–9) vs. 10 (6–13)], and SOFA scores [0 (0–2) vs. 2 (1–3)]. Of the 22 critical patients, 13 (59.1%) patients were discharged, 8 (36.4%) patients remained in hospital (Details in **Table 3**)

The radiological and echocardiography data on admission are summarized in **Table 4**. All patients had bilateral infiltrates on their chest CT scan. Twenty-one (32.3%) patients presented ground glass opacity (GGO) with critical patients more localized in the periphery and the center. Thirty patients

TABLE 2 | Laboratory findings of patients infected with COVID-19 on admission to ICU.

Variable	Normal range	ALL patients ($n = 37$)	Severe ($n = 15$)	Critically severe ($n = 22$)	p
Hemoglobin, g/L	115–150	127.0 (112–142)	128.0 (119–150)	126.0 (109–139)	0.202
White blood cell count, $\times 10^9$/L	3.5–9.5	7.8 (5.3–11.4)	5.6 (4.8–8.0)	9.3 (6.9–11.8)	0.135
Neutrophil count, $\times 10^9$/L	1.8–6.3	6.1 (3.3–9.4)	4.8 (2.4–7.1)	7.0 (4.6–9.6)	0.197
Lymphocyte count, $\times 10^9$/L	1.1–3.2	0.7 (0.5–1.0)	0.8 (0.6–1.3)	0.7 (0.5–1.0)	0.449
Monocyte count, $\times 10^9$/L	0.1–0.6	0.5 (0.3–0.7)	0.4 (0.3–0.7)	0.5 (0.3–0.9)	0.266
Platelet count, $\times 10^9$/L	125–350	207.0 (114–259)	253.0 (191–289)	179.0 (106–252)	0.005
D-dimer, mg/L	0–0.5	1.1 (0.7–1.7)	1.0 (0.7–1.2)	1.2 (0.8–1.8)	0.329
Creatine kinase, U/L	20–140	129.0 (63–258)	90.0 (52–244)	152.5 (69–355)	0.111
Creatine kinase–MB, U/L	0–16	9.0 (7–13.5)	8.0 (6–10)	11.0 (7.8–12.3)	0.062
Alanine aminotransferase, U/L	7–40	31.0 (21.5–61.5)	29.0 (24–42)	38.5 (20.8–69.5)	0.596
>40, *N* (%)		16 (43.2%)	5 (33.3%)	11 (50.0%)	0.315
Aspartate aminotransferase, U/L	13–35	41.0 (30.5–59.0)	35.0 (30–44)	50.0 (34.2–69.0)	0.060
>35, *N* (%)		22 (57.9%)	6 (40%)	16 (72.7%)	0.047
Gamma-glutamyl transferase, U/L	10–60	43.0 (26.0–96.5)	33 (24–95)	49.5 (26.8–101.0)	0.284
>60, *N* (%)		16 (43.2%)	5 (33.3%)	10 (45.5%)	0.460
Total bilirubin, mmol/L	0–20	13.0 (9–16)	14.0 (9–15)	11.5 (8.0–16.3)	0.655
Blood urea nitrogen, mmol/L	2.5–6.1	5.0 (3.9–6.0)	4.0 (3–5.1)	5.24 (4.4–6.9)	0.018
Creatinine, μmol/L	46–92	60.0 (55–76)	57.0 (52–63)	64.5 (57.5–80.8)	0.044
Hypersensitive troponin I, ug/L ≥0.015, No. (%)	0–0.015	3 (8.1%)	0	3 (13.6%)	0.202
Procalcitonin, ng/mL ≥0.05, No. (%)	<0.05	2 (5.4)	1 (6.7%)	1 (4.5%)	0.951
Brain natriuretic peptide, pg/ml	0–100	39.0	31.0 (14–58)	64.5 (15.0–156.0)	0.056
Lactate, mmol/L	0.7–2.1	2.4 (1.9–3.1)	2.8 (2.4–3.1)	2.2 (1.7–3.0)	0.143
Albumin, g/l		31.9 (29.1–34.6)	32.8 (30.5–34.9)	30.5 (28.5–33.9)	0.279
Total cholesterol, mmol/l		3.9 (3.5–4.6)	4.4 (3.7–5.2)	3.8 (3.4–4.4)	0.075
IL-2, pg/ml	<3.1	0.9 (0.6–1.1)	0.9 (0.6–1.1)	0.9 (0.7–1.1)	0.357
IL-4, pg/ml	<3.0	0.8 (0.6–1.4)	1.0 (0.5–1.4)	0.7 (0.6–1.4)	0.843
IL-6, pg/ml	<3.0	12.7 (3.49–80.2)	4.9 (2.5–52.4)	38.6 (4.6–103.1)	0.115
IL-10, pg/ml	<4.1	5.9 (3.6–14.6)	4.3 (3.1–7.1)	11.4 (3.7–18.5)	0.367
TNF-a, pg/ml	<3.1	0.2 (0.1–0.5)	0.2 (0.1–0.5)	0.2 (0.1–0.5)	0.716
T cell(CD3+), %	53.7–80.9	53.6 (42.5–66.3)	53.6 (41.3–64.1)	54.3 (42.5–67.9)	0.952
CD4+T cell, %	27.4–49.2	32.3 (27.8–40.1)	32.2 (28–40.2)	33.3 (27.4–40.3)	0.962
CD8+T cell, %	15.8–37.5	18.3 (12.5–27.1)	18.3 (10.9–28.0)	18.3 (13.4–24.5)	0.910
B cell, %	5.1–20.3	22.2 (14.2–29.4)	20.7 (12.8–24.9)	23.9 (14.8–40.8)	0.167
NK cell, %	6.7–30.9	13.9 (10.8–27.8)	18.7 (13.8–28.5)	12.9 (7.7–19.1)	0.069

Data are n (%) or median (IQR).

had echocardiography data. The most common abnormality in echocardiography was diastolic dysfunction and left atrial enlargement. All cases had normal cardiac output or increased cardiac output.

DISCUSSION

This retrospective cohort study presented severe and critical patients' clinical characteristics and outcomes in Wenzhou city who were hospitalized with the COVID-19 infection. In this study, we found higher age, and higher SOFA and APACHE II scores on admission were associated with disease severity. Additionally, elevated levels of Blood urea nitrogen, decreased levels of platelet were more common in critical COVID-19 patients. To our best knowledge, this retrospective cohort study is the first report to compare severe and critical patients from one city with COVID-19 outside Wuhan.

Comparing our data with those published from Wuhan, we found that patients in Wenzhou city had a milder infection (8, 9). According to our study, the epidemiological, demographic, symptom, and laboratory results, and CT scans of COVID-19 were similar to these previous studies (6, 7, 9). The announced mortality in Hubei is much higher, which shows a significant regional difference. Compared with female or those of a younger age, male, and people of an older age (>65 years) are more likely to develop ARDS (10). In previous studies, evidence found that older and male patients are the most susceptible to the COVID-19 infection, nearly 70% of patients infected by

TABLE 3 | Treatments and outcomes of patients infected with COVID-19.

Variable	ALL patients ($n = 37$)	Severe ($n = 15$)	Critically severe ($n = 22$)	*P*
Treatment				
Glucocorticoid therapy	21 (56.8%)	3 (20%)	18 (81.8%)	<0.001
Immunoglobulin therapy	19 (51.4%)	7 (46.7%)	12 (54.5%)	0.638
Thymosin	21 (56.8%)	4 (26.7%)	17 (77.3%)	0.002
Thaliduan	15 (40.5%)	4 (26.7%)	11 (50%)	0.156
Antiviral treatment	37	15	22	–
Antibiotic treatment	37	15	22	–
Oxygen treatment				
High flow	18 (48.6%)	0	18 (81.8%)	<0.001
NIV	9 (13..8)	0	9 (40.9%)	0.005
IMV	10 (15.4%)	0	10 (45.5%)	0.002
ECMO	6 (16.2%)	0	6 (27.3%)	0.063
Prone Position Ventilation	7 (18.9%)	0	7 (31.8%)	0.028
CRRT	3 (8.1%)	0	3 (13.0%)	–
Complications				
Sepsis	34 (91.9%)	12 (80%)	22 (100%)	0.059
Septic Shock	6 (16.2%)	0	6	0.063
ARDS	11 (29.7%)	0	11 (50%)	<0.001
Acute cardiac injury	17 (45.9%)	1 (6.7%)	16 (72.7%)	<0.001
Acute kidney injury	8 (21.6%)	1 (6.7%)	7 (31.8%)	0.156
Acute liver injury	33 (89.2%)	12 (80.0%)	21 (95.5%)	0.344
Secondary infection	13 (35.1%)	1 (6.7%)	12 (54.5%)	0.003
Acidosis	10 (27.0%)	0	10 (45.5%)	0.002
Prognosis				
28-day mortality	1 (2.7%)	0	1 (4.5%)	

Data are n (%); NIV, non-invasive ventilation; IMV, invasive mechanical ventilation; ECMO, extracorporeal membrane oxygenator; CRRT, continuous renal replacement therapy; ARDS, Acute respiratory distress syndrome.

COVID-19 were male (7, 11), which is also supported by our data. We observed that critical patients were significantly older than severe patients. Elderly patients experienced a markable decline in cell-mediated immune function and reduced human immune function. The induction of proinflammatory cytokines after infection is not adequately controlled by anti-inflammatory mechanisms in elderly persons, potentially leading to a poor prognosis (12).

Liver complications, including elevated levels of ALT, AST, or bilirubin are common in patients with sepsis. Several large-scale case studies have reported the clinical features of patients with COVID-19 (4, 6, 9). These data indicate that 14–53% of patients with COVID-19 reported increased levels of ALT and AST during disease progression. Patients with severe or critical COVID-19 seem to have higher rates of liver dysfunction. In our study, elevation of ALT or AST was observed in 22 (33.8%) out of 37 patients. In addition, gamma-glutamyl transferase (GGT), which is a diagnostic biomarker for cholangiocyte injury, has not been reported clearly. We found that GGT was elevated in 26 (70.3%) out of 37 patients with COVID-19 during hospitalization. Compared with severe cases, critical cases were more likely to have an elevation of AST, liver injury was also related to the disease severity. Angiotensin-converting enzyme 2 (ACE-2) is a membrane-bound aminopeptidase that has a vital role in the liver and immune systems (13). ACE-2 has been identified as a functional receptor for coronaviruses (14). The preliminary study by Chai et al. suggested that ACE-2 receptor expression is enriched in cholangiocytes, indicating that COVID-19 might directly bind to ACE-2-positive cholangiocytes to dysregulate liver function (15).

Based on previous studies, nearly 17% of patients had acute cardiac injury, with a high sensitive cardiac troponin I increase or abnormalities seen in electrocardiography and echocardiography (4, 8, 11). In our study, only 3 critical patients had hsCnTI mile increase on admission, showing a significant regional difference. Cardiac function was enhanced in the early stage and was less likely to develop into acute cardiac injury. The potential explanation of this difference may be early intervention. For critical patients, COVID-19 manifests as "silent hypoxemia," showing rapid deterioration and death after admission, which may begin the process of long-term hypoxia which can easily cause damage to myocardial cells. In Wenzhou, the government searched for suspected COVID-19 patients to be admitted to the hospital for treatment, we were capable enough to provide effective medical care to all infected patients to improve the prognosis of COVID-19.

The APACHEII and SOFA scores reflect the state and degree of illness severity and multi-organ dysfunction, respectively, the

TABLE 4 | Radiological and echocardiography data of patients infected with COVID-19.

Variable	ALL patients (*n* = 37)	Severe (*n* = 15)	Critically severe (*n* = 22)
Density			
GGO	21 (32.3%)	11 (66.7%)	10 (45.5%)
consolidation	1 (1.5%)	0	1 (4.5%)
mixed	15 (23.1%)	4 (27.6%)	11 (50.0%)
Location			
Peripheral	15 (40.5%)	9 (60.0%)	6 (27.3%)
Central and peripheral	22 (59.5%)	6 (40.0%)	16 (72.7%)
Pleural effusion	3 (4.6%)	2 (13.3%)	1 (4.5%)
Echocardiography, *N*	30	10	20
cardiac output, CO L/min	6.0 (1.6)	6.6 (1.6)	5.7 (1.5)
stroke volume, SV ml	75.4 (16.7)	79.6 (18.7)	73.4 (15.6)
Ejection fraction, EF %	63.5 (4.8)	66.0 (3.5)	62.3 (5.0)
Diastolic dysfunction	17 (56.7%)	7 (70%)	10 (50%)
pulmonary hypertension	2 (6.7%)	0	2 (10%)
Left atrial enlargement	17 (56.7%)	5 (50%)	12 (60%)
Mitral regurgitation	7 (23.3%)	0	7 (35%)
Tricuspid regurgitation	4 (13.3%)	0	4 (20%)

GGO, ground glass opacity; Data are n (%).

SOFA score is also a good diagnostic marker for sepsis (16, 17). High APACHEII and SOFA scores on admission can help clinicians to identify a patient's illness severity at an early stage. In previous studies, more than half of the patients developed sepsis. In addition, we found that sepsis is really common in severe and critically ill patients, but only several developed septic shock. Decreases in CD4+ T-cell levels, and lymphopenia and abnormal cytokine levels were common features in cases of COVID-19, which might be a critical factor associated with disease severity and mortality (18, 19). We observed a decrease in lymphocytes and a significant increase in cytokine IL-6 levels, but in our case cohort, only a few patients' CD4+ T-cell levels decreased. It shows that those patients had a milder overall condition and they also may have benefited from receiving timely and effective intervention and treatment, and their immune levels had not significantly decreased. Furthermore, more than half of the patients received immunoglobulin and Thymosin therapy to improve immunity.

Our study has some limitations. Firstly, several patients in our study still remain in the hospital, the final outcome is not complete, but those patients have survived longer than 28 days. Secondly, several patients were transferred from other medical institutions and may have received effective intervention before being transferred, this hospital admission may not be their first admission. Thirdly, only 37 patients were included, our conclusion might be limited by the sample size.

CONCLUSIONS

The mortality of critically ill patients with COVID-19 is low in our study. Cardiac function was enhanced in the early stage and therefore less likely to develop into acute cardiac injury, but most patients suffered with acute liver injury. The CT imaging presentations of COVID-19 in critical patients were mostly patchy ground glass opacities in the peripheral areas under the pleura, and more likely with consolidation, and bilateral lung involvement.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The study was approved by the First Affiliated Hospital of Wenzhou Medical University Ethics Committee.

AUTHOR CONTRIBUTIONS

J-YP: design of the study. S-ZQ and WH: collection of data. L-m: data management. C-l and Z-f: analysis. S-ZQ: wrote the paper. J-YP: critical revision of the article. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the National Natural Science Foundation of China (81671968), the Medical Innovation Discipline of Zhejiang Province (Critical Care Medicine, Y2015), and the Major project co-founded by Zhejiang Province and Ministry of Science and Technology (WKJ-ZJ-1909).

REFERENCES

1. Organization WH. *Director-General's Remarks at the Media Briefing on 2019-nCoV on 11 February 2020*. (2020). Available online at: https://www.who.int/dg/speeches/detail/who-director-general-s-remarks-at-the-media-briefing-on-2019-ncov-on-11-february-2020 (accessed February 12, 2020).
2. Xu XW, Wu XX, Jiang XG, Xu KJ, Ying LJ, Ma CL, et al. Clinical findings in a group of patients infected with the 2019 novel coronavirus (SARS-Cov-2) outside of Wuhan, China: retrospective case series. *BMJ*. (2020) 368:m606. doi: 10.1136/bmj.m606
3. Xu YH, Dong JH, An WM, Lv XY, Yin XP, Zhang JZ, et al. Clinical and computed tomographic imaging features of novel coronavirus pneumonia caused by SARS-CoV-2. *J Infect*. (2020) 80:394–400. doi: 10.1016/j.jinf.2020.02.017
4. Guan WJ, Ni ZY, Hu Y, Liang WH, Ou CQ, He JX, et al. Clinical characteristics of Coronavirus disease 2019 in China. *N Engl J Med*. (2020) 382:1708–20. doi: 10.1101/2020.02.06.20020974
5. Rello J, Tejada S, Userovici C, Arvaniti K, Pugin J, Waterer G. Coronavirus disease 2019 (COVID-19): A critical care perspective beyond China. *Anaesth Crit Care Pain Med*. (2020) 19:167–9. doi: 10.1016/j.accpm.2020.03.001

6. Yang W, Cao Q, Qin L, Wang X, Cheng Z, Pan A, et al. Clinical characteristics and imaging manifestations of the 2019 novel coronavirus disease (COVID-19):A multi-center study in Wenzhou city, Zhejiang, China. *J Infect.* (2020) 80:388–93. doi: 10.1016/j.jinf.2020.02.016
7. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet*. (2020) 395:497–506. doi: 10.1016/S0140-6736(20)30183-5
8. Yang X, Yu Y, Xu J, Shu H, Xia JA, Liu H, et al. Clinical course and outcomes of critically ill patients with SARS-CoV-2 pneumonia in Wuhan, China: a single-centered, retrospective, observational study. *Lancet Respir Med*. (2020) 8:475–81. doi: 10.1016/S2213-2600(20)30079-5
9. Wang D, Hu B, Hu C, Zhu F, Liu X, Zhang J, et al. Clinical characteristics of 138 hospitalized patients with 2019 novel coronavirus-infected pneumonia in Wuhan, China. *JAMA*. (2020) 323:1061–9. doi: 10.1001/jama.2020.1585
10. Arabi YM, Arifi AA, Balkhy HH, Najm H, Aldawood AS, Ghabashi A, et al. Clinical course and outcomes of critically ill patients with middle east respiratory syndrome coronavirus infection. *Ann Intern Med*. (2014) 160:389–97. doi: 10.7326/M13-2486
11. Chen N, Zhou M, Dong X, Qu J, Gong F, Han Y, et al. Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study. *Lancet*. (2020) 395:507–13. doi: 10.1016/S0140-6736(20)30211-7
12. Opal SM, Girard TD, Ely EW. The immunopathogenesis of sepsis in elderly patients. *Clin Infect Dis*. (2005) 41 (Suppl. 7):S504–12. doi: 10.1086/432007
13. Turner AJ, Hiscox JA, Hooper NM. ACE2: from vasopeptidase to SARS virus receptor. *Trends Pharmacol Sci*. (2004) 25:291–4. doi: 10.1016/j.tips.2004.04.001
14. Zheng YY, Ma YT, Zhang JY, Xie X. COVID-19 and the cardiovascular system. *Nat Rev Cardiol*. (2020) 17:259–60. doi: 10.1038/s41569-020-0360-5
15. Chai X, Hu L, Zhang Y, Han W, Lu Z, Ke A, et al. Specific ACE2 expression in cholangiocytes may cause liver damage after 2019-nCoV infection. *bioRxiv*. (2020). doi: 10.1101/2020.02.03.931766
16. Ferreira FL, Bota DP, Bross A, Mélot C, Vincent JL. Serial evaluation of the SOFA score to predict outcome in critically ill patients. *JAMA*. (2001) 286:1754–8. doi: 10.1001/jama.286.14.1754
17. Singer M, Deutschman CS, Seymour CW, Shankar-Hari M, Annane D, Bauer M, et al. The third international consensus definitions for sepsis and septic shock (Sepsis-3). *JAMA*. (2016) 315:801–10. doi: 10.1001/jama.2016.0287
18. Kohlmeier JE, Woodland DL. Immunity to respiratory viruses. *Annu Rev Immunol*. (2009) 27:61–82. doi: 10.1146/annurev.immunol.021908.132625
19. Hui H, Zhang Y, Yang X, Wang X, He B, Li L, et al. Clinical and radiographic features of cardiac injury in patients with 2019 novel coronavirus pneumonia. *medRxiv*. (2020). doi: 10.1101/2020.02.24.20027052

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Public Health

REVIEW
published: 04 September 2020
doi: 10.3389/fpubh.2020.551889

Iran's Approach to COVID-19: Evolving Treatment Protocols and Ongoing Clinical Trials

Ramin Rahmanzade[1,2†], *Reza Rahmanzadeh*[1,3†], *Seyed MohammadReza Hashemian*[1*‡] *and Payam Tabarsi*[1*‡]

[1] Chronic Respiratory Diseases Research Center (CRDRC), National Research Institute of Tuberculosis and Lung Diseases (NRITLD), Shahid Beheshti University of Medical Sciences, Tehran, Iran, [2] Faculty of Medicine, University of Basel, Basel, Switzerland, [3] Neurologic Clinic and Policlinic, Departments of Medicine, Clinical Research, and Biomedical Engineering, University Hospital Basel and University of Basel, Basel, Switzerland

OPEN ACCESS

Edited by:
Zisis Kozlakidis,
International Agency for Research on Cancer (IARC), France

Reviewed by:
Amgad Hanna,
University of Wisconsin-Madison, United States
Masoud Behzadifar,
Lorestan University of Medical Sciences, Iran

***Correspondence:**
Seyed MohammadReza Hashemian
iran.criticalcare@yahoo.com
Payam Tabarsi
Payamtabarsi@yahoo.com

[†]These authors share first authorship

[‡]These authors share senior authorship

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Public Health

Received: 23 April 2020
Accepted: 13 August 2020
Published: 04 September 2020

Citation:
Rahmanzade R, Rahmanzadeh R, Hashemian SM and Tabarsi P (2020) Iran's Approach to COVID-19: Evolving Treatment Protocols and Ongoing Clinical Trials. Front. Public Health 8:551889. doi: 10.3389/fpubh.2020.551889

The coronavirus disease 2019 (COVID-19) pandemic is challenging the health care systems around the world and compelling them to timely share their strategies, tactics and experiences. Since mid-January, a huge volume of instructions has been released by Iran's Ministry of Health and Medical Education (MOHME) covering diverse aspects of disease control and prevention. In this study, we aimed to review the instructions published either before or after COVID-19's transmission to Iran to depict the clinical approach and therapeutics used in Iran to battle the current pandemic. We retrospectively gathered and critically reviewed all official situation reports, guidelines, guidance, flowcharts, protocols, recommendations and advice released by Iranian scientific, or administrative arms of action against COVID-19. The ongoing clinical trials approved by MOHME and registered to the Iranian Registry of Clinical Trials (IRCT) have been reviewed as well. Our study resulted in the following mainstays of Iran's approach to COVID-19: (i) active clinical screening; preferably on-line or on-phone, (ii) management of limited paraclinical resources; by using them as diagnostic tools rather than epidemiological, (iii) a trend toward outpatient care of mild-to-moderate cases; either confirmed or suspicious, with active scheduled follow-up, and (iv) avoidance of pharmacotherapy, as far as possible. The therapeutic and administrative instructions are still being actively updated with some recommendations different from the previous ones. Nevertheless, a common approach in the background could be detected, It seems that the instructions are conceptually in line with the first "National Guideline for 2019-nCoV" published on 20 January 2020. The screening has mainly been clinically oriented rather than being based on laboratory tests and MOHME seems to be following the approach of "early detection of symptomatic cases followed by early source control."

Keywords: Iran, COVID-19, SARS-CoV-2, antiviral therapy, infection prevention and control, public health

INTRODUCTION

Coronavirus Disease 2019 (COVID-19) first detected as unusual pneumonia in four Chinese patients on 26 December 2019, was quickly declared by the World Health Organization (WHO) as "Public Health Emergency of International Concern" on 30 January and, finally, as Pandemic on 11 March 2020 (1, 2).

The microbial cause of COVID-19 was identified on 7 January to be a coronavirus, at first called novel Coronavirus 2019 (2019-nCoV) and later SARS-CoV-2 by WHO (1, 2).

To timely being prepared against a possible epidemic of the novel coronavirus, Center for Disease Control and Prevention (CDC) of Iran's Ministry of Health and Medical Education (MOHME) released a comprehensive actionable guideline, named "National Guideline for 2019-nCoV" on 20 January 2020 and established thereby the primary framework for prevention, early detection and treatment of patients in the onward outbreak of the novel coronavirus based on WHO's Risk Communication and Community Engagement (RCCE) strategies, risk management strategies, infection prevention and control (IPC) strategies and internal instructions (3). A new, more detailed edition was released on 2 February, while still no case of COVID-19 was detected in Iran (4).

On 19 February 2020, while COVID-19 was reported in a total of 75,204 cases from 26 countries, MOHME officially announced the death of two old patients due to COVID-19 in Iran (5). Five days later, MOHME established the "Scientific Committee of COVID-19" aimed to release and continuously update an actionable "Diagnostic Therapeutic Flowchart for COVID-19," abbreviated hereinafter as DTFC, as an appendix to the above-mentioned National Guidelines for 2019-nCoV. As of 6 June 2020, the flowchart that was first released on 25 February (DTFC1) has been updated six times (DTFC2-7) with several changes according to the national and international experiences (6–11).

Furthermore, specific guidance and protocols have been released for the clinical management of patients with COVID-19 in intensive care units (ICU) and for the pediatric and pregnant population (12–14).

In the past month, MOHME announced the appearance of second country-wide wave of COVID-19, which emerged in the regions spared by the first wave and spread to previously affected areas.

A summary of the main actions of WHO, MOHME, and "Iran's National Headquarter Against COVID-19" (INHAC) and a timeline of national and international events is outlined in **Table 1**.

Of note, the present narrative review is independent research trying to identify Iran's strategies against the pandemic of SARS-CoV-2 through scientific review of official documents released by responsible authorities.

Abbreviations: WHO, world health organization; 2019-nCoV, novel coronavirus 2019; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; COVID-19, Coronavirus Disease 2019; RCCE, Risk Communication and Community Engagement; IPC, infection prevention and control; SCC-19, Scientific Committee of COVID-19; DTFC, Diagnostic Therapeutic Flowchart for COVID-19; INHAC, Iran's National Headquarter Against COVID-19; MOHME, Iran's Ministry of Health and Medical Education; RT-PCR, reverse transcriptase- polymerase chain reaction; ICU, intensive care unit; INF, interferon; SpO2, saturation of peripheral oxygen; PaO2, partial pressure of oxygen.

IRAN'S INTERNAL GUIDELINES, FLOWCHARTS, AND PROTOCOLS ON COVID-19: A SUMMARY OF MAIN POINTS

Since mid-January, different instructions and recommendations covering diverse aspects of the disease control and prevention have been released by INHAC, MHOME and their subdivisions in order to minimize the burden of the disease and the speed of its propagation.

The authors retrospectively gathered and critically reviewed all official situation reports, guidelines, guidance, flowcharts, protocols, recommendations and advice. The documents were collected through search of all actual or archived official documents released by MOHME or INHAC on the web since 26 December 2019.

Although the instructions are still being actively updated with some recommendations different from the previous ones, a common approach in the background could be detected. As noted on the cover page of DTFCs, these flowcharts are subjected to revisions based on new scientific findings or upcoming resource limitations.

The mainstays of the current approach consist of (i) Active clinical screening; preferably on-line or on-phone (ii) Management of limited paraclinical resources; by using them as diagnostic tools rather than epidemiological (iii) A trend toward outpatient care of mild-to-moderate cases; either confirmed or suspicious, with active scheduled follow-up (iv) Pharmacotherapy should be avoided as far as possible, rather in hospitalized patients or those who have been defined as high risk population.

Active Clinical Screening

In January 2020, MOHME mainly tried to inform people about symptoms of COVID-19 and encourage patients with respiratory symptoms, especially those with a history of recent travel to China, to seek medical attention not too late. This approach of "early detection of symptomatic cases followed by early source control" was reinforced by the release of the National Guideline of 2019-nCoV on 20 January. Since late January, moreover, all passengers have been subjected to temperature screening upon arriving in Iran.

Despite these preparations, the transmission of the novel coronavirus was officially announced on 19 February and has been attributed to some passengers from China, who visited the crowded city Qom, which is one of the two main religious towns of the country. Of note, the visa-free policy of Iran for Chinese citizens may facilitate the travel and entry of Chinese to Iran at the beginning of pandemic in China.

Since 6 March 2020, the screening for COVID-19 has been entered a new phase. On this day, MOHME launched the "National Campaign Against COVID-19" and, thereby, established the three following bases for screening: (i) An electronic simple-to-deal portal, in which people fulfill a short web-based questionnaire with 6 questions. Finally, they receive a notification noting the possibility of having COVID-19

TABLE 1 | Timeline of main responses to the novel coronavirus pandemic at national and international level.

27. Dec 2019	1st report of 4 unusual pneumonia to local CDC in Wuhan, China
31. Dec 2019	1st report of pneumonia of unknown cause to the WHO China Country Office
04. Jan 2020	WHO publicly announced the pneumonia of unknown causes in Wuhan, China on social media
05. Jan 2020	WHO's 1st disease outbreak news advised against travel and trade restriction with China
07. Jan 2020	Novel coronavirus (nCoV-2019) identified
10. Jan 2020	- WHO's released "National capacities review tool for a novel coronavirus": ongoing active monitoring and preparedness - WHO published an "Advice for international travel and trade": no restriction for international traffic
12. Jan 2020	China publicly shared the genetic sequence of 2019-nCoV
13. Jan 2020	- The 1st reported case of COVID-2019 outside of China (in Thailand) - WHO published an interim guidance for Risk communication and community engagement (RCCE), readiness and response to the novel coronavirus (2019-nCoV); updated on 26 January and 16 March 2020
17. Jan 2020	WHO released interim guidance for "laboratory testing for 2019-nCoV" (last update on 19 March)
20. Jan 2020	- MOHME released Iran's National Guideline for 2019-nCoV - WHO's field visit to Wuhan - WHO released an interim guidance for "home care of mild patients" (last update on 17 March 2020)
21. Jan 2020	China publicly released primers and probes used in rRT-PCR kits
22. Jan 2020	WHO's mission to China observed evidences of human-to-human transmission
24. Jan 2020	WHO published an update of "Advice for international travel and trade": Advice for exit screening in countries with ongoing transmission and entry screening in countries without transmission
26. Jan 2020	Iran started screening at Point of Entry (PoE)
28. Jan 2020	- WHO released an interim guidance of Clinical management of severe acute respiratory infection (SARI) when 2019-nCoV is suspected—1st report of limited human-to-human transmission outside China
30. Jan 2020	WHO declared the outbreak a Public Health Emergency of International Concern
01. Feb 2020	Iran's government officially banned flights from China
02. Feb 2020	MOHME released the 2nd edition of Iran's National Guideline for 2019-nCoV
05. Feb 2020	Iran's government repatriated Iranian nationals from Wuhan, China (They have been isolated and closely monitored by MOHME)
11. Feb 2020	- WHO named the novel virus and the disease, SARS-CoV-2 and COVID-19, respectively - WHO convened a Research and Innovation forum on COVID-19 - WHO published key consideration for repatriation of travelers
19. Feb 2020	MOHME officially announced death of two patients due to COVID-19 in Iran
21. Feb 2020	MOHME release a guidance on environmental sanitation and safe burial
22. Feb 2020	- MOHME established the Scientific Committee of COVID-19 (SCC-19) - Iran's government closed schools and universities in the provinces affected by COVID-19
23. Feb 2020	Iran's government established Iran's National Headquarter against COVID-19
25. Feb 2020	SCC-19 released a "Diagnostic Therapeutic Flowcharts for COVID-19" (DTFC1)
26. Feb 2020	Interim guidance for IPC of COVID-19 in pregnant or breastfeeding mothers and in neonates and infants with mothers confirmed or suspicious to COVID-19
27. Feb 2020	Some provinces in Iran started to clinically screen the travelers at PoE
28. Feb 2020	Iran's National Headquarter against COVID-19 closed all schools around the country, decreased working hours and announced a nation-wide screening of travelers at PoE of all cities
02. Mar 2020	- WHO's field visit to Iran -SCC-19 released a DTFC for pediatric population - MOHME released an operative guidance for drug delivery and follow up in out-patient setting - MOHME officially advised against routine use of corticosteroid in COVID-19 - MOHME temporarily decreased the frequency of prenatal care visits
03. Mar 2020	- SSC-19 released DTFC2 and a protocol for management of critically ill patients with COVID-19 in intensive care units (ICU) - MOHME released interim guidance for follow up of COVID-19 patients treated in out-patient setting - MOHME released an action plan for care of COVID-19 patients in convalescent care facilities - MOHME released a guidance for in-patient care of pregnant women, confirmed or suspicious to COVID-19
04. Mar 2020	- MOHME launched a National public awareness campaign and released a detailed action plan
06. Mar 2020	- MOHME launched a National campaign against COVID-19 for active screening of COVID-19 and released a detailed action plan
07. Mar 2020	- MOHME released various therapeutic guidance for management of COVID-19 in patients with underlying chronic diseases e.g., cancer - MOHME released nutritional guidance for patients with COVID-19, treated in out-patient or in-patient settings - MOHME released guidance for telerehabilitation during viral epidemic
10. Mar 2020	- SCC-19 released DTFC3 - MOHME released a guidance for the use of Iranian traditional medicines in patients with COVID-19 - MOHME updated the operative guidance for drug delivery and follow up in out-patient setting, first released on 2 March 2020

(Continued)

TABLE 1 | Continued

11. Mar 2020	- WHO characterized COVID-19 as a pandemic
12. Mar 2020	- WHO's expert mission to Iran acknowledged Iran's strategies and comprehensive coordinated approach against COVID-19
13. Mar 2020	- WHO updated the interim guidance of Clinical management of SARI when 2019-nCoV is suspected, first released on 28. Jan 2020 - WHO launched COVID-19 Solidarity Response Fund
15. Mar 2020	- MOHME officially advised against routine use of oseltamivir in COVID-19 - MOHME released an additional protocol for management of COVID-19 in ICU to the previous guidance released on 03. Mar 2020
18. Mar 2020	- WHO launched the Solidarity Trial (2) - SCC-19 released DTFC4 - MOHME released an operative guidance for home care management of patients with mild COVID-19
25. Mar 2020	SCC-19 released DTFC5
30. Mar 2020	MOHME updated the action plan for care of COVID-19 patients in convalescent care facilities, first released on 3 March 2020
04. Apr 2020	MOHME released a guidance for clinical trials related to COVID-19
10. Apr 2020	MOHME launched the 2nd phase of National campaign against COVID-19: follow up of cases detected in 1st step and close-contact tracing of confirmed cases
14. Apr 2020	MOHME released an update of the protocol for management of critically ill patients with COVID-19 in intensive care units (ICU)
29. Apr 2020	SCC-19 released DTFC6
28. Jun 2020	SCC-19 released DTFC7

In addition to the mentioned guidelines, flowcharts and protocols a significant number of advices, recommendations and guidance regarding environmental sanitation, social distancing, mental health and infection prevention and control (IPC) have been released by MOHME and other aligned, responsible authorities.

and some related recommendations. Moreover, in the case of being clinically suspicious to COVID-19 or being in contact with known cases of COVID-19, they will be automatically called by determined healthcare providers responsible for patients geographical sub-region, (ii) Telephone Screening through regional and sub-regional healthcare authorities via some publicly announced phone numbers, and (iii) Screening offices at selected and publicly announced medical centers and hospitals (15).

As of 5 August 2020, MOHME clinically screened more than 90% of Iran's population with above-mentioned methods as opposed to a total of 2,560,374 persons who were screened by the reverse transcriptase-polymerase chain reaction (RT-PCR) test.

As discussed above, the screening in Iran has mainly been clinically oriented and symptoms-based rather than being purely based on laboratory tests, an approach in agreement with the above-mentioned approach of "early detection of symptomatic patients followed by early source control." However, getting equipped with enough laboratory resources e.g., diagnostic RT-PCR kits has activated the laboratory-based screening for a broader population of suspicious cases. On 10 April, MOHME launched the second phase of National Campaign Against COVID-19, mainly aimed to screen the people in close-contact with confirmed cases uning RT-PCR tests.

Management of Limited Paraclinical Resources

The National Guidelines for 2019-nCoV mention that specimens, either from the upper or lower respiratory tract, should be taken from suspicious cases. Moreover, the RT-PCR should be repeated every 3–4 days in hospitalized patients until having two negative results with a time interval of at least 24 h. The first update of DTFC (DTFC2), released on 3 March 2020, mentioned that the RT-PCR for the detection of E gene (screening test) must be done for all hospitalized patients suspicious to COVID-19 and should be avoided in the outpatient setting. In addition, RT-PCR for the detection of N gene (confirmation test) should only be ordered in intubated patients with a positive RT-PCR for the E gene. The Next update (DTFC3), released on 10 March 2020, added that sampling and testing in the outpatient setting could be done in immunocompromised patients and health care providers suspicious to COVID-19.

Performing the RT-PCR test in clinically suspicious patients was first explicitly mentioned in DTFC6. It seems that the parallel increase in laboratory resources and reduction in the number of cases caused the extension of the eligible populations. In this line, all patients in close-contact with confirmed cases should also be tested based on DTFC6.

Blood tests of C-reactive protein (CRP) and complete blood count (CBC) have been noted that could be measured in afebrile patients without dyspnea presenting with respiratory symptoms, in the case of suspicion to COVID-19. However, DTFC6 substituted RT-PCR tests for these blood tests in clinically suspicious patients in the outpatient setting. DTFC7 restricted RT-PCR tests in outpatient setting only to patients or people with close contact (with a known COVID-19 case) who are older than 60 years-old, pregnant, or with a known medical history, which is a risk factor for COVID-19. Beside, based on DTFC7 all hospitalized cases need to perform RT-PCR test.

The role of imaging examinations as useful tools in the diagnosis of COVID-19 was not discussed until the DTFC1 was released. It could partly be resulted from the characteristic pulmonary involvement of COVID-19 compared to the SARS and MERS, which has been reported by other affected countries, especially China.

As shown in **Figure 1**, pulmonary imaging should be exploited in (a) febrile high-risk patients, defined in **Figure 1**, without dyspnea and (b) immunocompromised patients with clinical suspicion of COVID-19, independent of being febrile or not. The

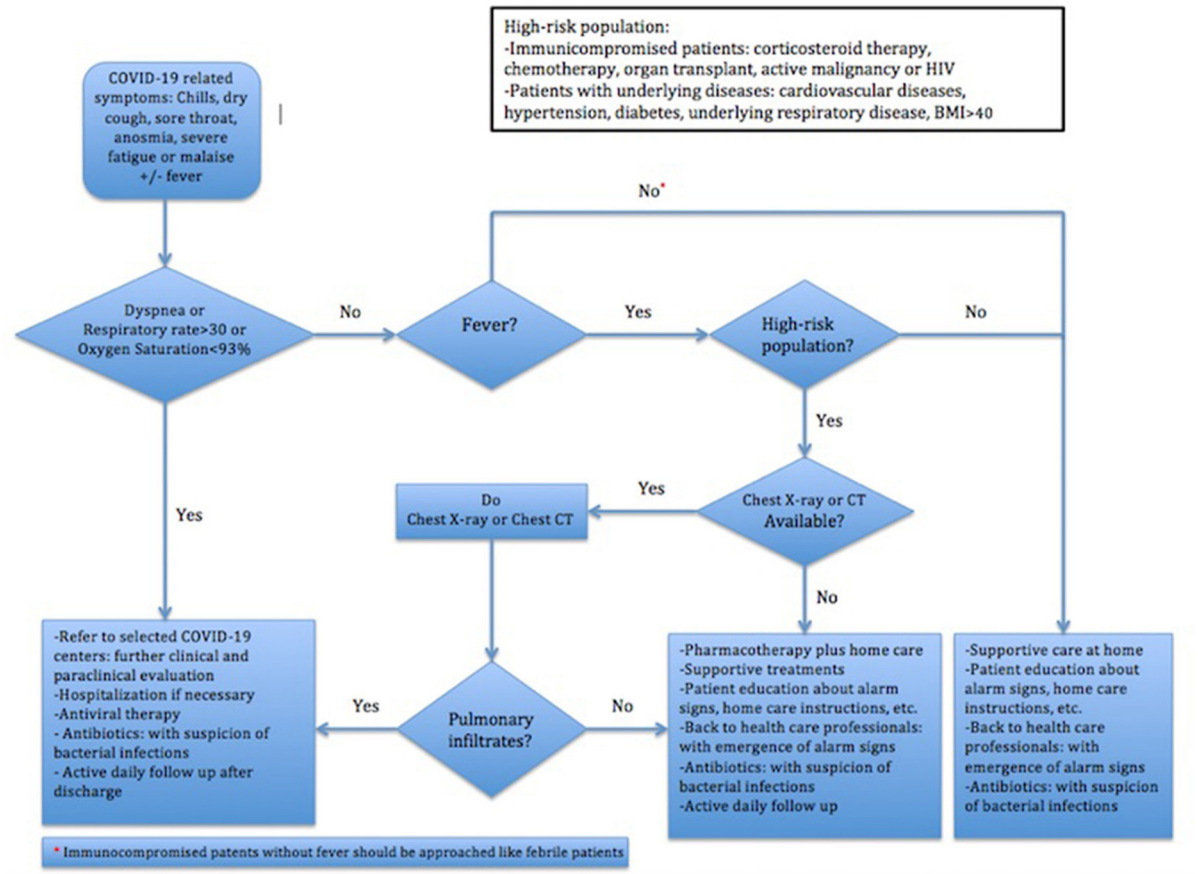


FIGURE 1 | Iran's Flowchart of Clinical Approach to COVID-19 (10).

second group has been considered first in DTFC2. According to the DTFC2 and the later updates, COVID-19 compatible findings in chest CT scans might act as equivalent to the RT-PCR test in confirmation of the diagnosis of COVID-19 in clinically suspicious patients.

Although lung imaging has not been still considered as a tool in the follow-up of patients, a significant resolution of pulmonary involvement in imaging was included as an indispensable criterion for patient discharge from hospital in DTFC3-5, which has been removed in DTFC6.

A Trend Toward Outpatient Care

The first guideline released on 20 January emphasized that patients with the mild disease could be cared at home and should be consulted to back to COVID-19 centers for early evaluation upon exacerbation of symptoms. In this line, DTFCs strongly recommended home care in all patients except the followings: (a) those presenting with respiratory symptoms having dyspnea or respiratory rate $> = 30$ per min or oxygen saturation $<93\%$, (b) high-risk febrile patients with imaging findings compatible with COVID-19, and (c) immunocompromised patients suspicious to COVID-19. According to the "Operative guidance for home care management of patients with mild COVID-19," patients should be consulted to enough rest, appropriate meals, high fluid intake and isolation. A detailed brochure, provided by MOHME, containing information about patient care at home, instructions of drug consumption, alarming symptoms and isolation will be given as well. In addition, all patients receiving outpatient care or therapy should be isolated for at least 14 days after the resolution of symptoms.

An active follow-up has been considered in all DTFCs as an indispensable part of the care of COVID-19 patients in outpatient settings. The follow-up is generally composed of (1) continuous self-monitoring and (2) scheduled surveillance by health care providers. According to the DTFC1, all patients treated in the outpatient setting should be daily followed up by health care providers in on-phone mode and upon emerging one of the following symptoms should be referred to hospitals: (a) dyspnea, (b) loss of consciousness, (c) continuation of fever, and (d) exacerbation of cough. However, the two latter criteria have been revised in DTFC2 to (c) a continuation of fever after 5 days from treatment start and (d) exacerbation of cough or productive cough, which are still valid.

The follow-up takes place through the Iranian Integrated Health portal, the so-called SIB®. The duration of daily telephone follow-up recommended by DTFC1 and DTFC2 has

been explained in the next updates more detailed. According to the DTFC3-5, active telephone follow-up should daily be done in the first 5 days accomplished by a final follow-up 10 days after being registered as suspicious COVID-19 patient in SIB®. In DTFC6, the telephone follow-up should be done every 2 days until 14 days after registration in SIB®. In this regard, operative guidance was released on 2 March 2020, updated on 10 March, to precisely address the mechanism of drug delivery and follow-up in the outpatient setting. Based on these instructions, all patients receiving outpatient therapy will be registered in SIB® and have to be followed up as recommended in DTFCs.

Notably, convalescent care facilities have found a place in the care of patients with COVID-19 as an intermediate stage between inpatient and outpatient care settings and the role of these centers has been well-defined in an action plan released on 3 March 2020, updated later on 30 March 2020. Accordingly, patients with positive RT-PCR results, who: (a) are not hospitalized or (b) have been discharged earlier than 14 days after symptom onset or (c) might not receive enough care at home, should be referred to these centers.

Therapeutics in Outpatient and Inpatient Settings: Regular Reconsideration

The national guideline released on 20 January 2020 and its single update on 2 February 2020, did not deal with therapeutics in detail. They generally recommended oxygen therapy, conservative rehydration and empirical antibiotic therapy in the setting of severe acute respiratory infection (SARI). Moreover, they are strongly against the routine use of corticosteroid in COVID-19 and recommend, also, the use of oseltamivir only when influenza is suspected. However, DTFCs have been mainly devised to be exploited as ready-to-use clinical action plans.

All versions of DTFC emphasized that COVID-19 could be managed in most patients without special antiviral or antibiotic therapies. In the outpatient setting, the recommendation of adjunctive medicines with possible beneficial effects against SARS-CoV-2 has been restricted to the following patient groups presenting with respiratory symptoms: (a) febrile high-risk patients without dyspnea with normal lung imaging (b) immunocompromised patients without dyspnea with normal lung imaging; independent of being febrile or afebrile.

In DTFC1-3, the treatment regimen in the outpatient setting consisted of oseltamivir 75 mg and hydroxychloroquine sulfate 200 mg (or chloroquine phosphate 250 mg) both twice daily for a minimum of five and a maximum of 14 days. In DTFC4-6 similar to the National Guidelines for 2019-nCoV, the indication of oseltamivir prescription has been restricted to those, in whom there is a virological or epidemiological clue of influenza infection.

In DTFC5-6, the proposed daily dose of hydroxychloroquine sulfate (or chloroquine phosphate) has been doubled on the first day of therapy and, also, the maximum duration of therapy has been reduced to 10 days. In DTFC7, the dosage for hydroxychloroquine sulfate (or chloroquine phosphate) administration remained the same as DTFC6, however was restricted to so-called "high risk population."

In the inpatient setting, two different treatment regimens based on disease severity were proposed in DTFC1-4. According to the DTFC1-4, COVID-19 is considered to be very severe if at least one item of the followings is present: (a) loss of consciousness, (b) respiratory rate >= 24 per min, (c) systemic blood pressure < 90/60 mm Hg, (d) multilobular infiltration on lung imaging, (e) persistent hypoxemia.

In patients not being classified as very severe, a combination of (i) hydroxychloroquine sulfate 200 mg (or chloroquine phosphate 250 mg) twice daily only on the 1st day, (ii) lopinavir/ritonavir 200 mg/50 mg two tablets twice daily for a minimum of five and a maximum of 14 days, which based on DTFC3-6 could be replaced by atazanavir/ritonavir 300/100 in the case of gastrointestinal intolerance or past history of cardiac arrhythmia. If atazanavir/ritonavir is prescribed, the hydroxychloroquine sulfate 200 mg twice daily should be continued for 5–14 days, (iii) oseltamivir 75 mg twice daily for 5–14 days was also recommended in DTFC1-3, not recommended in the later updates.

In patients, in whom the disease course was classified as very severe, ribavirin 1,200 mg daily based on DTFC1-2 and 2,400 mg daily based on DTFC3-4 plus the above-mentioned regimens was recommended. However, ribavirin has been removed from the proposed regimen in DTFC5-6. DTFC7 does not recommend hydroxychloroquine sulfate (or chloroquine phosphate) anymore for inpatient setting and recommends: (i) lopinavir/ritonavir 200/50 mg two tablets twice daily or atazanavir/ritonavir 300/100 once daily for a minimum of seven and a maximum of 14 days (ii) interferon-beta-1-a 250 microgram or interferon-beta-1-a 44 microgram subcutaneous every other day 5–7 days.

Indeed, the DTFC5-7 do not deal anymore with critically ill patients and referred the clinical management of this group of patients to a separate "protocol for the management of critically ill patients with COVID-19 in intensive care units (ICU) (12)."

Therefore, DTFC5-7 recommends only hydroxychloroquine sulfate 200 mg (or chloroquine phosphate 250 mg) two tablets twice daily on the 1st day and then one tablet twice daily for 7–14 days in the inpatient setting. In addition, concomitant therapy with lopinavir/ritonavir or atazanavir/ritonavir (two tablets twice daily for 7–14 days) is a dispensable part of standard regimen in DTFC5-7 and might be ordered at the discretion of the responsible clinicians.

According to the ICU protocol, lopinavir/ritonavir or atazanavir/ritonavir should be prescribed in ICU-admitted patients with a respiratory rate >= 24 per min or SpO_2 =< 80–85% (12). This protocol recommended ventilatory support suggested by WHO (16), the Surviving Sepsis Campaign (17), and Marini and Gattinoni (18). It considers oxygen therapy if SpO_2 <90% and recommends intubation in COVID-19 patients as early as one item of the followings is present: (a) persistent hypoxemia (PaO_2 < 60 mmHg or SpO_2 < 85%) following 1–2 h application of non-invasive ventilation or 30–60 min usage of high-flow devices (b) moderate to severe respiratory acidosis ($PaCO_2$ >= 60 mmHg or PH = < 7.25) (c) Respiratory rate >= 36 per minute (d) Hemodynamic instability (mean arterial pressure (MAP) < 60 mmHg without response to the fluid therapy), and (e) loss of consciousness.

REGISTERED CLINICAL TRIALS

As of 4 August 2020, a total of 305 clinical trials have been registered to the Iranian Registry of Clinical Trials (IRCT) (19). As a part of WHO's SOLIDARITY trial, 16 centers in Iran are involved in a large five-arm randomized controlled trial with a target sample size of 3,000, The recruitment phase has been completed in May 2020. SOLIDARITY trial (IRCT20200405046953N1) aims to evaluate the safety and efficacy of four different medicines including Remdesivir, chloroquine/hydroxychloroquine, lopinavir/ritonavir, and interferon plus lopinavir/ritonavir on COVID-19. The enrolled patients will receive these medications in conjunction with the local standard regimens.

Among registered trials, 17 randomized trials evaluate or compare the safety and efficacy of different antivirals including sofosbuvir, sofosbuvir/ledipasvir, sofosbuvir plus velpatasvir, sofosbuvir plus daclatasvir, ribavirin, lopinavir/ritonavir, favipiravir, umifenovir, and remdesivir in the treatment of COVID-19 (IRCT20151227025726N14, IRCT20200322046833N1, IRCT20200128046294N2, IRCT20200324046850N2, IRCT20200318046812N1, IRCT20100228003449N29, IRCT20171122037571N2, IRCT20130812014333N145, IRCT20200328046882N1, IRCT20200421047155N1, IRCT20130812014333N145, IRCT20080901001165N46, IRCT20200403046926N1, IRCT20200328046886N1, IRCT20200406046968N3, IRCT20180725040596N2, IRCT20200428047228N1). The largest randomized trial on antivirals (IRCT20200318046812N1) is currently recruiting patients from 11 centers with a target sample size of 324. It is designed to compare the therapeutic efficacy of hydroxychloroquine plus favipiravir with a combination of hydroxychloroquine plus lopinavir/ritonavir in COVID-19.

A single-arm non-controlled trial (IRCT20171122037571N2) with a target sample size of 120 evaluates the safety and efficacy of remdesivir in COVID-19 patients. The control group is treated with the standard regimen and the intervention group concomitantly receives remdesivir and the standard regimen for 5 days. The recruitment phase has been completed in May 2020.

As the humoral immunity is vastly involved in antiviral immunity, some groups suggested the potential beneficial role of blood products in the treatment of COVID-19. Eighteen trials have been designed to evaluate the efficacy and safety of convalescent plasma or intravenous immunoglobulin in COVID-19 (IRCT20200325046860N1, IRCT20200310046736N1, IRCT20151228025732N53, IRCT20181104041551N1, IRCT20200325046859N1, IRCT20200317046797N3, IRCT20200409047007N1, IRCT20200413047056N1, IRCT20200416047099N1, IRCT20200406046968N2, IRCT20200501047258N1, IRCT20200418047116N1, IRCT20150808023559N21, IRCT20200414047072N1, IRCT20120215009014N353, IRCT20200404046948N1, IRCT20150808023559N20, IRCT20200328046882N1). The largest of them (IRCT20200325046860N1), is currently recruiting patients with severe COVID-19 from four COVID-19 centers with a target sample size of 200 and an expected recruitment completion on 20 August 2020. A trial with a target sample size of 45 (IRCT20200310046736N1) aims to compare the safety and efficacy of convalescent plasma with the plasma-derived immunoglobulin-enriched solution in COVID-19 patients.

Seven trials have been registered to assess the efficacy of interferon (INF) ß-1a or 1b (IRCT20080901001165N53, IRCT20200406046968N3, IRCT20160118026097N3, IRCT20190804044429N1, IRCT20150914024017N1, IRCT20100228003449N28, IRCT20100228003449N27, IRCT20151227025726N12). The recruitment phase of the four latters has been completed with a targeted sample size of 40, 30, 30 and 20, respectively. A trial (IRCT20190804044429N1) with a sample size of 70 compares the efficacy of the standard regimen with the combination of the standard regimen and INF ß-1b. The recruitment phase completed in May 2020. The largest one (IRCT20080901001165N53) with a sample size of 100 is studying the efficacy of INF ß-1a nasal spray.

Among 10 registered trials in the recruiting phase, which evaluate the prophylactic or therapeutic efficacy of chloroquine/hydroxychloroquine in COVID-19 (IRCT20130917014693N10, IRCT20100228003449N30, IRCT20120826010664N6, IRCT20200718048129N1, IRCT20100228003449N3, IRCT20130306012728N8, IRCT20080901001165N51, IRCT20190122042450N4, IRCT20151222025660N2, IRCT20200405046958N1), the first one is the largest with a target sample size of 100 patients and an expected date of recruitment completion of 8 June 2020. The latter (IRCT20080901001165N51), a randomized trial, studies the efficacy of hydroxychloroquine nasal spray in 80 patients.

Nine trials are aimed to study the safety and efficacy of mesenchymal stem cell therapy in COVID-19, all with a small sample size of 5–10 (IRCT20200418047121N2, IRCT20200413047063N1, IRCT20130306012728N8, IRCT20190717044241N2, IRCT20200217046526N2, IRCT20200217046526N1, IRCT20140911019125N6, IRCT20200325046860N2, IRCT20140528017891N8). The recruitment phase of the two latters with a sample size of 5 and 10 has been completed.

Five trials (IRCT20151227025726N13, IRCT20150303021315N17, IRCT20200501047258N1, IRCT20200406046968N3, IRCT20130306012728N8) evaluate the safety and efficacy of Tocilizumab in COVID-19. The latter, a multicentric study with a target sample size of 500 will expectedly complete the recruitment phase in July 2020.

The use of corticosteroids in COVID-19 is still controversial. WHO's interim guidance released on 13 March 2020 and Iran's National guidelines for 2019-nCoV are explicitly against routine use of corticosteroids (3, 4, 16).

To evaluate the efficacy of corticosteroids, six trials have been registered (IRCT20200324046852N1, IRCT20200404046947N1, IRCT20200204046369N1, IRCT20200406046963N1, IRCT20170210032478N3, IRCT20120215009014N354). These are randomized trials investigating efficacy of corticosteroid administration, either oral or inhalational or intravenous, as adjunctive therapy to the standard regimens.

The recruitment phase of the two first trials with a sample size of 30 and 40 patients, respectively, has been completed.

A large multicentric trial (IRCT20200318046812N2), with a target sample size of 906, recruits as of 10 April patients from four centers for 1 year. This study aims to evaluate the safety

and efficacy of three therapeutic interventions with the following regimens: "Hydroxychloroquine + Azithromycin + Naproxen + Prednisolone" or "Hydroxychloroquine + Azithromycin + Naproxen" regimens or "Hydroxychloroquine + Kaletra."

ACE2, a negative regulator of the renin-angiotensin-aldosterone system (RAAS) works as the SARS-CoV-2 entry receptor. In this line, medications with activity on RAAS e.g., ACE inhibitors and angiotensin receptor blockers (ARB) could be of therapeutic interest.

A randomized controlled trial (IRCT20180802040678N4) with a target sample size of 100 patients evaluates the efficacy of losartan 25 mg twice daily on the treatment of COVID-19. The recruitment phase has been completed in May 2020. Similar to SARS-CoV, the spike protein priming of the SARS-CoV-2 during entry to the alveolar epithelial cells occurs through the serine protease TMPRSS2 (20). A randomized clinical trial (IRCT20200317046797N1) comparing a treatment group receiving camostat mesylate, a serine protease inhibitor, plus the standard regimen with a control group receiving only the standard regimen completed its recruiting phase in May 2020.

A total of 98 clinical trials has been registered to the IRCT to evaluate the efficacy of diverse herbal or traditional Iranian medicines, supplements or vitamins e.g., high dose Vit C (IRCT20190917044805N2) and Vit D3 (IRCT20200324046850N1).

As of 5 August 2020, a total of 314,786 confirmed, 17,617 death and 272,535 recovered COVID-19 cases have been reported by Iran.

CONCLUSION

The presented narrative review tries to identify the mainstays of Iran's approach to COVID-19. As almost all of the national instructions released by responsible authorities are in Persian, this review may contribute to the global data sharing in the era of COVID-19. The presented review found that the instructions are conceptually in line with the first "National Guideline for 2019-nCoV" published on 20 January 2020. The screening has mainly been clinically oriented rather than being purely based on laboratory tests and MOHME seems to be following the approach of "early detection of symptomatic cases followed by early source control."

AUTHOR CONTRIBUTIONS

RaR devised the project and the main conceptual idea. RaR and ReR gathered, reviewed the documents, took the lead in writing the manuscript, and wrote it in consultation with SH and PT. SH and PT revised the first draft of manuscript. RaR drew the diagram. ReR, SH, and PT made the table. All authors proofed the final draft of manuscript.

REFERENCES

1. Wu Z, McGoogan JM. Characteristics of and important lessons from the coronavirus disease 2019 (COVID-19) outbreak in China: summary of a report of 72 314 cases from the Chinese center for disease control and prevention. *JAMA*. (2020) 323:1239–42. doi: 10.1001/jama.2020.2648
2. WHO Timeline-COVID-19. Available online at: https://www.who.int/news-room/detail/08-04-2020-who-timeline---covid-19.
3. Iran's National Guideline for 2019-nCoV; 1st edition. Available online at: https://firoozgar.iums.ac.ir/files/baharestan/files/dam.pdf.
4. Iran's National Guideline for 2019-nCoV; 2nd edition. Available online at: https://firoozgar.iums.ac.ir/files/baharestan/files/kesh.pdf.
5. WHO's Situation Report-30. Available online at: https://www.who.int/docs/default-source/coronaviruse/situation-reports/20200219-sitrep-30-covid-19.pdf?sfvrsn=3346b04f_2.
6. Iran's Diagnostic Therapeutic Flowchart for COVID-19; 1st edition (DTFC1). Available online at: https://irimc.org/Portals/0/NewsAttachment/%20%20%20%20%20%20%20.pdf.
7. Iran's Diagnostic Therapeutic Flowchart for COVID-19; 2nd edition (DTFC2). Available online at: https://firoozgar.iums.ac.ir/files/baharestan/files/ftb.pdf.
8. Iran's Diagnostic Therapeutic Flowchart for COVID-19; 3rd edition (DTFC3). Available online at: https://irimc.org/Portals/0/NewsAttachment/08_%20%20%20%20%20%20%20%20%20%2020%20.pdf.
9. Iran's Diagnostic Therapeutic Flowchart for COVID-19; 4th edition (DTFC4). Available online at: https://irimc.org/Portals/0/Images/News/%20نسخه%20چهارم%2028%20اسفند.pdf.pdf.
10. Iran's Diagnostic Therapeutic Flowchart for COVID-19; 5th edition (DTFC5). Available online at: https://firoozgar.iums.ac.ir/files/baharestan/files/n5f.pdf.
11. Iran's Diagnostic Therapeutic Flowchart for COVID-19; 6th edition (DTFC5). Available online at: http://iranpath.org/files/site1/files/درمان_تشخیص_فلوچارت_ششم_نسخه.pdf.
12. *Iran's Protocol for Management of Critically ill Patients With COVID-19 in Intensive Care Units*. Available online at: http://treatment.sbmu.ac.ir/uploads/covid_19-ICU-4th_edition-Final.pdf.
13. *Iran's Diagnostic Therapeutic Flowchart for COVID-19 for Pediatric Population*. Available online at: http://treatment.sbmu.ac.ir/uploads/2-2-COVID-19.CHILD.second_version.pdf.
14. *Iran's Interim Guidance for IPC of COVID-19 in Pregnant or Breastfeeding Mothers and in Neonates and Infants With Mothers Confirmed or Suspicious to COVID-19*. Available online at: http://treatment.sbmu.ac.ir/uploads/10-1-etelaiye_3-madar_v_nozad_.pdf.
15. Iran's National Campaign Against COVID-19. Available online at: http://treatment.sbmu.ac.ir/uploads/dastoor_basij_meli.pdf.
16. *WHO's Interim Guidance of Clinical Management of Severe Acute Respiratory Infection (SARI) When 2019-nCoV is Suspected*. Available online at: https://apps.who.int/iris/handle/10665/330893.
17. Alhazzani W, Møller MH, Arabi YM, Loeb M, Gong MN, Fan E et al. Surviving sepsis campaign: guidelines on the management of critically ill adults with coronavirus disease 2019 (COVID-19). *Intensive Care Med*. (2020) 46:854–87. doi: 10.1007/s00134-020-06022-5
18. Marini JJ, Gattinoni L. Management of COVID-19 respiratory distress. *JAMA*. (2020) 323:2329–30. doi: 10.1001/jama.2020.6825
19. Iranian Registry of Clinical Trials (IRCT). Available online at: https://www.irct.ir/search/result?query=COVID-19.
20. Hoffmann M, Kleine-Weber H, Schroeder S, Krüger N, Herrler T, Erichsen S, et al. SARS-CoV-2cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically-proven protease inhibitor. *Cell*. (2020) 181:271–80.e8. doi: 10.1016/j.cell.2020.02.052

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Medicine

ORIGINAL RESEARCH
published: 08 September 2020
doi: 10.3389/fmed.2020.558539

The Relationship Between Chest Imaging Findings and the Viral Load of COVID-19

Wei Zhao[1], *Lei He*[2], *Haoneng Tang*[3], *Xingzhi Xie*[1], *Lingli Tang*[3]* and *Jun Liu*[1,4]*

[1] Department of Radiology, The Second Xiangya Hospital, Central South University, Changsha, China, [2] Department of Radiology, The First People's Hospital of Yueyang, Yueyang, China, [3] Department of Laboratory Medicine, The Second Xiangya Hospital, Central South University, Changsha, China, [4] Department of Radiology Quality Control Center, Changsha, China

Purpose: We aimed to investigate the relationship between clinical characteristics, radiographic features, and the viral load of patients with coronavirus disease 2019 (COVID-19).

Methods and Materials: We retrospectively collected 56 COVID-19 cases from two institutions in Hunan province, China. The basal clinical characteristics, detail imaging features and follow-up CT changes were evaluated and the relationship with the viral load was analyzed.

Results: GGO (48, 85.7%) and vascular enlargement (44, 78.6%) were the most frequent signs in COVID-19 patients. Of the lesions, 64.3% of the margins were uneasily differentiated. However, no significant correlations were found in terms of leucocytes, neutrophils, lymphocytes, platelets, and C-reactive protein (all $P > 0.05$). In contrast, the uneasily differentiated margin was negatively correlated with the *Ct* value ($r = -0.283$, $P = 0.042$), that is, an uneasily differentiated margin indicated a lower *Ct* value ($P = 0.043$). Patients with a lower *Ct* value were likely to present a progress follow-up change ($P = 0.022$). The *Ct* value at baseline could predict a progress follow-up change with an AUC of 0.685 (Cut-off value = 29.48). All four patients with normal CT findings presented new lesion(s) on follow-up CT scans.

Conclusion: The viral load of COVID-19 is negatively correlated with an uneasily differentiated lesion margin on initial CT scan images and the *Ct* value should noted when making a diagnosis. In addition, following-up CT scans are necessary for patients who presented a normal CT at the initial diagnosis, especially for those with a low *Ct* value.

Keywords: CT, COVID-19, viral load, follow-up, margin

OPEN ACCESS

Edited by:
Nicola Petrosillo, Istituto Nazionale per le Malattie Infettive Lazzaro Spallanzani (Scientific Institute for Research, Hospitalization and Healthcare), Italy

Reviewed by:
Chuanming Li, Chongqing Medical University, China
Ming Li, Fudan University, China
Hai Bo Xu, Wuhan University, China

***Correspondence:**
Lingli Tang
linglitang@csu.edu.cn
Jun Liu
junliu123@csu.edu.cn

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: *05 May 2020*
Accepted: *20 August 2020*
Published: *08 September 2020*

Citation:
Zhao W, He L, Tang H, Xie X, Tang L and Liu J (2020) The Relationship Between Chest Imaging Findings and the Viral Load of COVID-19. Front. Med. 7:558539. doi: 10.3389/fmed.2020.558539

KEY RESULTS

- The uneasily differentiated margin was negatively correlated with the *Ct* value ($r = -0.283$, $P = 0.042$). In another words, an uneasily differentiated margin indicated a lower *Ct* value.
- Follow-up CT scans are necessary for patients with normal CT findings at initial diagnosis, especially for those with a low *Ct* value.

INTRODUCTION

A cluster of "unknown viral pneumonia" cases in Wuhan, China, was reported to World Health Organization (WHO) on December 31 2019 (1). A novel coronavirus, named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was identified through deep sequencing analysis (2). The outbreak of coronavirus disease 2019 (COVID-19), declared as a public health emergency of international concern (PHEIC) (3), has raised intense concerns around the world (1). The situation of the outbreak of COVID-19 in China has been brought under control (4), however, it still threatens the global medical system.

The genome sequence findings suggested that the presence of COVID-19 was closely related to another coronavirus termed severe acute respiratory syndrome (SARS)-related CoV (5). According to the latest study (6), the modality of COVID-19 is lower than that of SARS-CoV. It has been proven that the possibility of human-to human transmission (7, 8) and the R_0 (*i.e.*, the expected number of additional cases that one case will generate) ranges from 2 to 3 (9). Since the pathogenesis and the many comprehensive biological features (*i.e.*, the microenvironment change and immune system reaction) of COVID-19 remain undiscovered, no specific antiviral agent and effective vaccine is available for treatment of this disease (10). Early detection, early diagnosis, early isolation, and early therapy remain the basic and essential strategies (11). Accurately assessing the disease severity of COVID-19 is still vital for clinical treatment scenarios and taking action in advance to avoid the presence of rapid progress. The viral load, inversely correlated with the cycle threshold (*Ct*) value, is considered as a parameter to reflect the disease severity (12–14) and indicate the transmission ability (15). However, not all hospitals reported the *Ct* value and only gave a binary diagnosis (*i.e.*, positive or negative). Moreover, the assessment of the *Ct* value of the virus needs a real-time reverse transcription-polymerase chain reaction test (RT-PCR), which has inherent disadvantages including possible false positive results and a long turnaround time. Identifying the potential clinical alternative factors of the *Ct* value may help us assess the disease severity efficiently.

Several available clinical factors, such as white blood cell/neutrophil/lymphocyte count, might have the potential to reflect the severity of COVID-19 (6, 8, 16). The clinical importance of computed tomography (CT) is emphasized by the evidence of its value in the screening, diagnosis, and evaluation for the daily treatment of patients with COVID-19 in clinical practice (17–19). Moreover, the radiographic features are also reported to reflect the severity of COVID-19 (17–19). Therefore, all the aforementioned potential risk factors may throw light on the viral load indirectly and be considered as convenient and alternative factors to reflect the condition of COVID-19. However, the relationship between the aforementioned risk factors with viral load remains unclear.

In the present research, the purpose is to investigate the relationship between clinical characteristics, radiographic features, and *Ct* values in patients with COVID-19 and provide some hints for its early diagnosis.

TABLE 1 | Clinical features, laboratory tests in our cohort.

Basal characteristics	All patients ($n = 56$)
Sex	
Male	26
Female	30
Age (years)[a]	50.34 ± 15.65
Epidemic history	
Direct exposure history[b]	21 (37.5)
Indirect exposure history	37 (66.1)
No exposure history	4 (7.1)
Family outbreak	17 (30.3)
Onset symptoms	
Fever	36 (64.3)
Cough	31 (55.4)
Myalgia or fatigue	10 (17.9)
Sore throat	6 (10.7)
Headache	5 (8.9)
Dyspnea	4 (7.1)
Diarrhea	2 (3.6)
Nausea and vomiting	1 (1.8)
More than one symptom	35 (62.5)
None	2 (2)
Underlying disease	
Cardiovascular and cerebrovascular diseases	3 (5.4)
Surgery history	1 (1.8)
Digestive system disease	3 (5.4)
Respiratory system disease	3 (5.4)
Endocrine system disease	2 (3.6)
None	45 (80.4)
Leucocytes ($\times 10^9$ per L)[c]	4.83 (3.57–6.24)
Neutrophils ($\times 10^9$ per L)	2.92 (2.35–4.18)
Lymphocytes ($\times 10^9$ per L)	1.08 (0.77–1.43)
Platelets ($\times 10^9$ per L)	163.50 (121–202)
C-reactive protein (mg/L)	14.95 (4.22–39.04)
Ct value	33.20 (28.30–36.66)

[a]presented as mean ± SD, [b]presented as number (percentage), [c]presented as median (Inter quartile range).

MATERIALS AND METHODS

This retrospective study was approved by our Medical Ethical Committee (Approved Number. 2020002), which waived the requirement for patients' informed consent referring to the CIOMS guideline.

Patients

In the study, we retrospectively included confirmed COVID-19 cases from Hunan Province, China. From January 16 2020 to February 6 2020, a search of the electronic system and the picture achieving and communication system (PACS) was performed to collect clinical features, laboratory values (the first one upon admission), epidemic characteristics, and all scanned CT images. The inclusion criteria included: (1) patients with PCR-confirmed

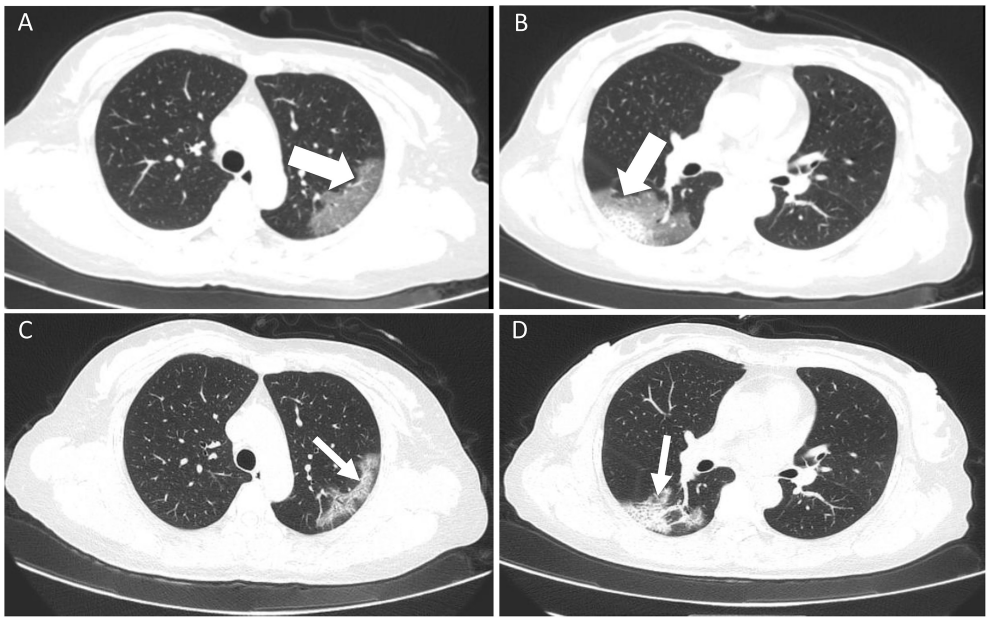


FIGURE 1 | A 52-year old female with confirmed COVID-19 infection. Patient had close contact with a confirmed case and the onset symptom of fever. **(A,B)** Initial CT scan (performed on February 5 2020) showed bilateral GGO and mixed GGO and consolidation (white thick arrow) with an easily differentiated margin. The viral load (*Ct* value) was 38.65. **(C,D)** The follow-up CT scan (performed on February 9 2020) showed an improvement change. All the lesions had been absorbed (white fine arrow).

COVID-19; (2) patients who underwent CT scanning before treatment; (3) the interval between a CT scan and throat swab sample being taken was <2 days; (4) the initial viral load was reported. The exclusion criteria included: (1) patients without PCR-confirmed COVID-19; (2) patients that had not undergone CT scanning before treatment; (3) the interval between a CT scan and throat swab sample being taken was more than 3 days; (4) No viral load was reported. Finally, 56 of 360 cases (30 women, 26 men; mean age, 50.34 years ± 15.65 [SD]; age range, 2–79 years) were included (27 patients from the Second Xiangya Hospital and 29 patients from the First People's Hospital of Yueyang). We characterized patients into four groups, mild type, common type, severe type, and fatal type based on the guideline of COVID-19 (Trial Version 7) (20), proposed by the China National Health Commission. Based on the different treatment regimens, we divided the included patients into two groups, non-severe group (mild type and common type) and severe group (severe type or fatal type). The interval between the onset of the disease and CT scans was 5 (2–8), presented as the median (Inter quartile range).

PCR Method

Duplex RT-PCR assays were performed by using throat swab samples in accordance with the protocol established by WHO (21). The nucleic acid was extracted by using an automatic system (Nathch CS, sansure biotich, Hunan). The nucleic acid amplification was performed on slan96P (Shanghai Hongshi Medical Technology Co., LTD). Each reaction tube was internally controlled. The *Ct* value was recorded for all samples and a *Ct* value <40 and >0 was considered as PCR positive.

Imaging Technique and Imaging Interpretation

All CT scans were performed with the following three scanners: Somatom definition AS (Siemens Medical Solutions), Somatom emotion (Siemens Medical Solutions), and ANATOM 16HD (ANKE Medical Solutions). The acquisition parameters were as follows: 120 kVp; 100–200 mAs; pitch, 0.75–1.5; and collimation, 1–5 mm, respectively. All imaging data were reconstructed by using a medium sharp reconstruction algorithm with a thickness of 1 mm. CT images were acquired in the supine position at full inspiration for all patients. All chest CT scans were reviewed blindly and independently by two radiologists (with 5 and 15 years of experience). If an inter-observer difference happened, the two radiologists would re-review the imaging feature(s) together and reach an agreement (in consensus). All images were viewed on both lung (width, 1,500 HU; level, −700 HU) and mediastinal (width, 350 HU; level, 40 HU) settings. Twelve imaging features including features of ground-glass opacities (GGO), consolidation, mixed GGO and consolidation, margin of the lesion (easily differentiated and uneasily differentiated, based on the lesions-lung interface), architectural distortion, reticulation, traction bronchiectasis, sub-pleural bands, intrathoracic lymph node enlargement, fibrosis, vascular enlargement in the lesion, and pleural effusions were evaluated according to our previous studies (18, 22). The number of involved lung lobes, the craniocaudal distribution (upper lung predominant, lower predominant, and no craniocaudal distribution), the transverse distribution (central or peripheral or no transverse distribution), and the scattering distribution (focal, multifocal, or diffuse) were also evaluated. The transverse distribution of the abnormalities

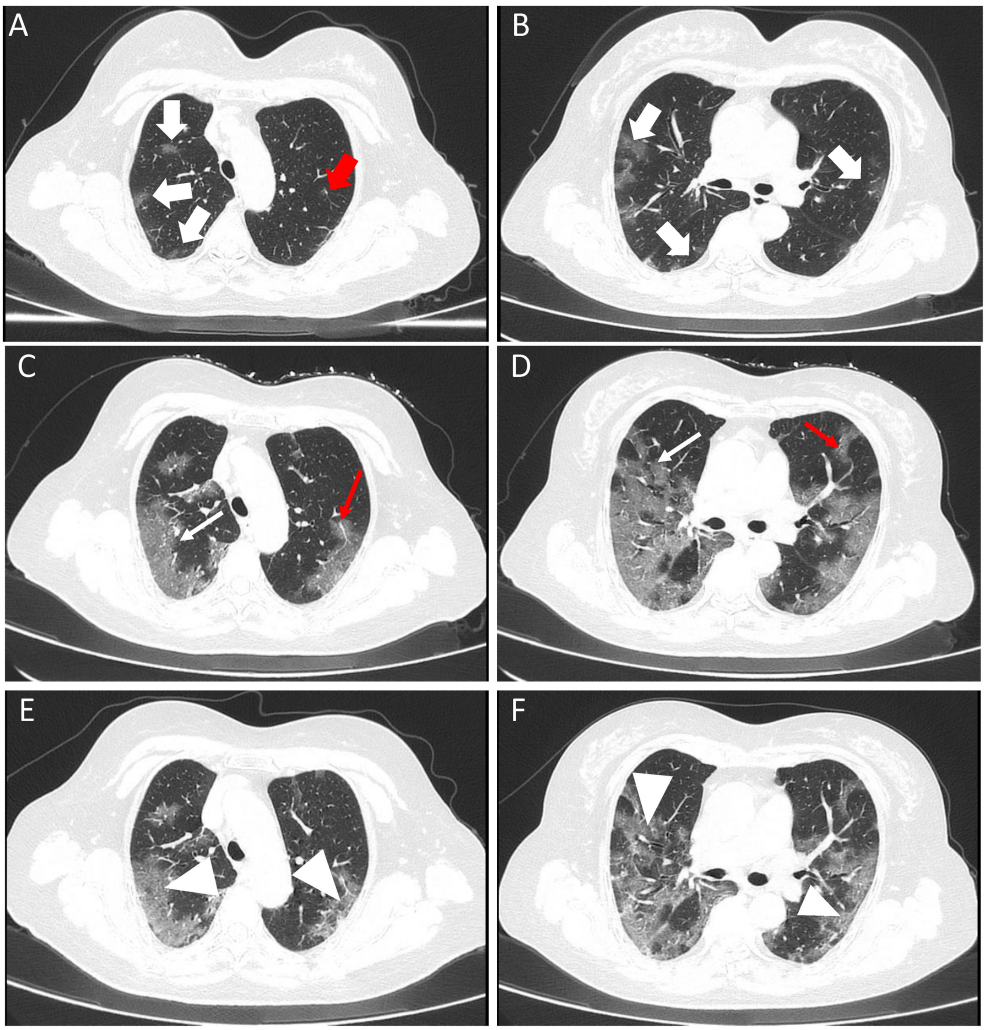


FIGURE 2 | A 67-year old female with confirmed COVID-19 infection. Patient had close contact with a confirmed case and the onset symptom of fever. **(A,B)** Initial CT scan (performed on January 31 2020) showed bilateral GGOs (white thick arrow) with an uneasily differentiated margin. The viral load (*Ct* value) was 29.13. **(C,D)** The first follow-up CT scan (performed on February 5 2020) showed a progress change. All the lesions had been enlarged (white fine arrow). The diameter of the vascular was larger than that of initial CT image [red fine arrow of **(C)**] and a new lesion was presented [red fine arrow of **(D)**]. Please note that the margin of lesions on first follow-up CT images was clearer than before. The second follow-up CT scan (performed on February 8 2020) showed an improvement change (white arrowhead) **(E,F)**.

were categorized as central (i.e., peribronchovascular), peripheral (i.e., sub-pleural), or with no transverse predilection. Focal was defined as a single lesion of abnormality, multifocal as more than one lesions, and diffuse as involvement of most of the volume of one lung lobe. A CT score system was used to evaluate the extent of disease (23). We defined three imaging changes: no change, progress change, and improvement change (22). No change referred to no obvious changes presented in the chest CT. Progress change referred to the presence of new lesions or the presence of an extent involvement area during the treatment. Improvement change referred to continually absorbed abnormities.

Statistical Analysis

Continuous variables were presented as median (IOR) and categorical variables were presented as numbers (%). The correlations between clinical features, laboratory tests, imaging features, and viral load were analyzed using the Spearman analysis. The ROC analysis was used to investigate the performance of the *Ct* value in predicting the follow-up change. A two-sided $P < 0.05$ was considered statistically significant. All statistical analyses were performed using the SPSS software (version 24.0).

RESULTS

Clinical Characteristics and Laboratory Detection

In the beginning, 4 patients (male 1, female 3) were divided into the mild group, 49 patients (male 23, female 26) were common, another 3 patients (male 2, female 1) were in the severe group. Twenty-one (37.5%) patients had a direct exposure history link

TABLE 2 | Imaging finds of patients with COVID-19.

Imaging findings	All patients ($n = 56$)
GGO	48 (85.7)
Vascular enlargement	44 (78.6)
Margin (uneasily differentiated)	37 (66.1)
Reticulation	26 (46.4)
Traction bronchiectasis	26 (46.4)
Consolidation	24 (42.9)
Fibrosis	22 (39.3)
Mixed GGO and consolidation	21 (37.5)
Architectural distortion	18 (32.1)
Sub-pleural bands	13 (23.2)
Pleural effusions	1 (1.8)
Intrathoracic lymph node enlargement	0 (0)
Craniocaudal distribution	
Upper lung predominant	6 (10.7)
Lower lung predominant	19 (33.9)
No craniocaudal distribution	27 (48.2)
Transverse distribution	
Central	0 (0)
Peripheral	46 (82.1)
No transverse distribution	6 (10.7)
Scattering distribution	
Focal	2 (3.6)
Multifocal	32 (57.1)
Diffuse	18 (32.1)
CT score	6 (3–7.75)
Number of involved lung lobe	
0	4 (7.1)
1	2 (3.6)
2	6 (10.7)
3	6 (10.7)
4	16 (28.6)
5	22 (39.3)
Number of lesions	
>5	40 (71.4)
<5	16 (28.6)
Number of absent CT findings	4 (7.1)
Follow-up CT changes	
Improvement change	27 (51.8)
Progress change	25 (41.1)

Data were presented as numbers (percentage), except for CT score, which presented as median (Inter quartile range).

to Wuhan (*i.e.,* long-term exposure history to Wuhan, traveling in Wuhan before diagnosis), 37 (66.1%) patients had an exposure history to confirmed patients. It is noted that 4 (7.1%) patients denied any direct exposure history and indirect exposure to confirmed patients and 17 (30.3%) patients were related to a family outbreak (more than 2 patients were confirmed in one family). Fever (36 of 56, 64.3%) and cough (31 of 56, 55.4%) were the most common onset symptoms. Other onset symptoms, including myalgia or fatigue, sore throat, dyspnea, diarrhea, nausea, and vomiting were presented in **Tables 1**, **2** patients had no onset symptoms and most patients (80.4%) had no underlying disease. The information about laboratory tests are also presented in **Table 1**. The median *Ct* value was 33.20 in our cohort.

TABLE 3 | The correlations between clinical features, laboratory tests and imaging features and viral load.

Characteristics	*Ct* value	
	r	*P*
Sex	0.181	0.183
Age	0.086	0.529
Leucocytes ($\times 10^9$ per L)	0.087	0.521
Neutrophils ($\times 10^9$ per L)	0.148	0.277
Lymphocytes ($\times 10^9$ per L)	−0.071	0.603
Platelets ($\times 10^9$ per L)	0.058	0.672
C-reactive protein (mg/L)	0.096	0.483
GGO	0.208	0.123
Vascular enlargement	0.094	0.490
Margin (reference: easily differentiated)	−0.298	**0.026**
Reticulation	−0.024	0.859
Traction bronchiectasis	−0.095	0.485
Consolidation	0.051	0.707
Fibrosis	0.152	0.265
Mixed GGO and consolidation	0.01	0.94
Architectural distortion	−0.05	0.716
Sub-pleural bands	−0.071	0.605
Pleural effusions	0.104	0.444
Craniocaudal distribution	0.158	0.246
Transverse distribution	0.081	0.555
Scattering distribution	0.135	0.322
CT score	0.179	0.187
Number of involved lung lobe	0.256	0.057
Number of lesions	0.125	0.360
Number of absent CT findings	0.227	0.092
Follow-up CT changes (reference: improvement change)	−0.322	**0.016**

The bold numbers indicated an significant correlation.

CT Findings

GGO (48 of 56, 85.7%) and vascular enlargement (44 of 56, 78.6%) were the most frequent signs in COVID-19 patients (**Figures 1**, **2**). The lesion's margins were 64.3% uneasily differentiated. Intrathoracic lymph node enlargement and pleural effusions were rare findings in our cohort. Lesions were more likely to be peripherally distributed (46 of 56, 82.1%) and contain bilateral involvement (49 of 56, 87.5%). 39.3% of patients had 5 lung lobes involved and 71.4% of patients had more than 5 lesions. Other evaluated imaging features are described in detail in **Table 2**. The median CT score of the lung involvement was 6. It is notable that 4 patients had no obvious abnormity on initial CT images.

The Relationships Between Clinical Factors, Imaging Findings and *Ct* Value

We investigated the relationships between clinical factors, imaging findings, and *Ct* value. No significant correlations

were found in terms of leucocytes, neutrophils, lymphocytes, platelets, and C-reactive protein (all $P > 0.05$). In contrast, the uneasily differentiated margin was negatively correlated with the *Ct* value ($r = -0.298$, $P = 0.026$, **Table 3**), that is, an uneasily differentiated margin indicated a lower *Ct* value, which potentially indicated a more severe presentation of the disease (**Figures 1**, **2**).

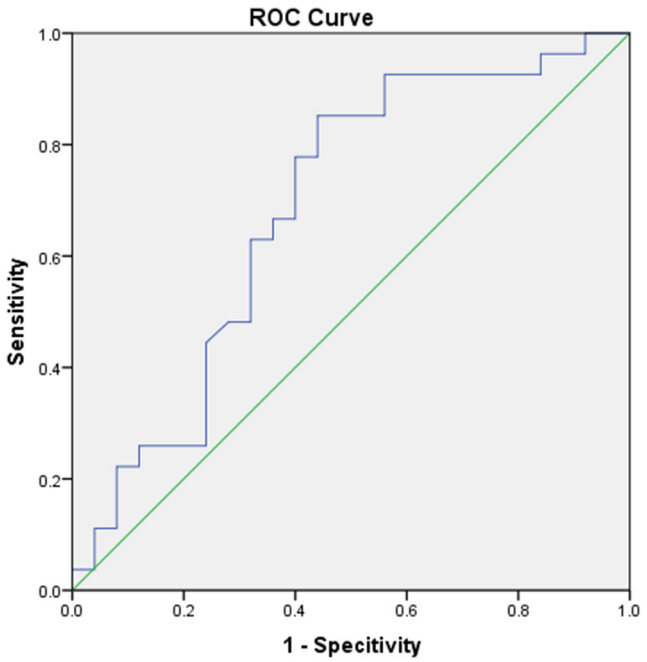


FIGURE 3 | The ROC curve of Ct value in predicting the follow-up CT changes.

Follow-Up CTs and The Relationship With *Ct* Value

In total, 52 of 56 (92.9%) patients had undergone follow-up CT scans. Among the 52 patients, 27 patients presented an improved change, whereas 25 patients presented a progressed change. Furthermore, we investigated the relationships between the follow-up CT changes and *Ct* value. The results showed that the progressed follow-up change was negatively correlated with the *Ct* value ($r = -0.322$, $P = 0.016$, **Table 3**), that is, patients with a lower *Ct* value were likely to present a progressed follow-up change ($P = 0.022$). The *Ct* value at baseline could predict a progress follow-up change with an AUC of 0.685 (Cut-off value = 29.48) (**Figure 3**). All 4 of the patients (*Ct* value: 25.23, 29.37, 25.22, and 33.19, respectively), with normal CT findings presented new lesion(s) on follow-up CT scans (**Figure 4**).

DISCUSSION

In the present study, we investigated the relationships between clinical characteristics, radiographic features, and *Ct* values in patients with COVID-19 and we found that an uneasily differentiated margin of lung lesions was negatively correlated with the *Ct* value, which could be used as a predictor for the

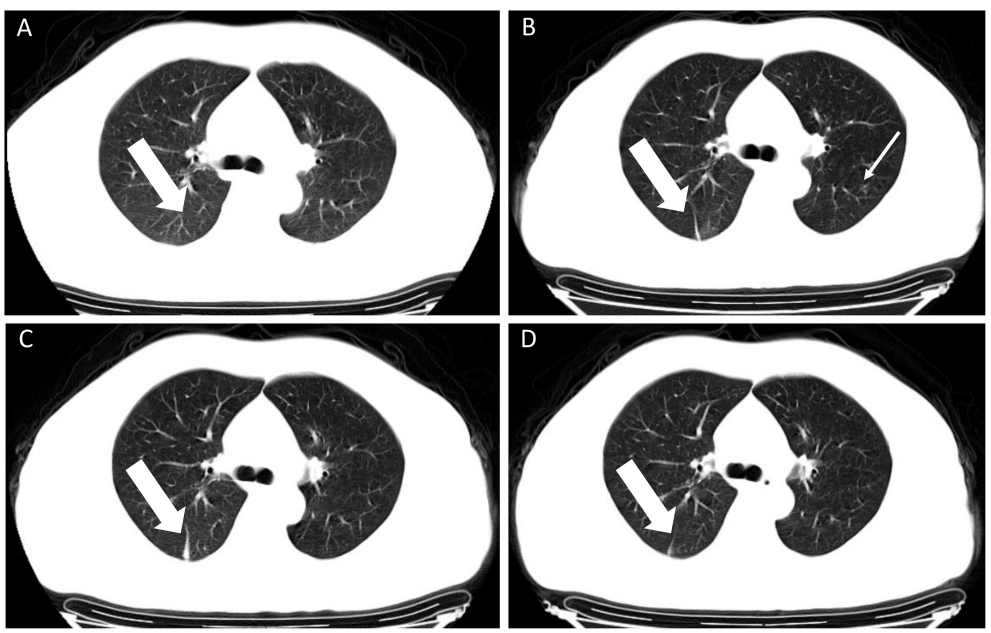


FIGURE 4 | A 45-year old male with confirmed COVID-19 infection. Patient had a direct exposure history to Wuhan and the onset symptom of vomiting. **(A–D)** CT scan performed four times. Initial CT scan (performed on January 30 2020) showed no obvious abnormal CT findings **(A)**. The next three times follow-up CT images showed a strip lesion in the right lower lobe (white thick arrow), first presented on second CT scan [performed on February 3 2020, **(B)**], enlarged on the third CT scan [performed on February 6 2020, **(C)**] and absorbed on the fourth CT scan [performed on February 9 2020, **(D)**]. An ambiguous lesion was shown in the upper left lobe [white fine arrow, **(B)**] and was absent in other images. The viral load (*Ct* value) was 22.53.

severity of COVID-19, that is, patients with a lower *Ct* value were likely to present a progress follow-up change ($P = 0.022$).

Both the number of confirmed cases and deaths has overtaken that of SARS in China (24). The clinical features and epidemic history have been well-reported recently. The onset symptom of fever and specific exposure history were also reported in our study. Most patients (66.1%) had an indirect exposure history and 17 (30.3%) patients were related to a family outbreak. The incidence indicated a serious risk of human-to-human transmission, therefore, early identification of positive cases and separating the negative patients from the suspected group is urgently warranted.

Although advances in treatment scenarios have been made, there is no existing evidence of curative medicine for COVID-19. Early diagnosis and treatment remained the basic strategies. The treatment response and clinical outcome of patients with COVID-19 were not well-documented, especially for severe/fatal patients or patients with rapid progress, so identifying patients with potential rapid progress early, accurately evaluating the severity of the disease at baseline, and further predicting clinical outcomes may improve the prognosis and curative rate. It was reported that the viral load (*Ct* value) has the potential to determine the severity of the disease (14). However, obtaining the viral load needs a long-term PCR test which has the potential of providing a false negative (18), therefore, investigating the relationships between these factors and the vial load may overcome the disadvantage. Leukopenia, lymphopenia, thrombocytopenia, and elevated C-reactive protein (CRP) levels were identified as risk factors for severe cases (6, 8). Liu et al. has discovered that the *Ct* value of the virus highly correlates with CRP and lymphopenia in patients with COVID-19. However, no laboratory manifestations were correlated with the viral load which may contribute to data bias, given the fact that we included a relatively large sample size.

CT scans, most frequently used in the diagnosis and monitoring treatment response of COVID-19, has contributed a lot in clinical practice. The typical chest CT features have been reported in previous studies (17, 25). GGO was the most frequent sign among the positive patients in our study, which is consistent with previous studies (25). In addition, the radiographic features were also considered as predictors for the severity of the disease (6). The CT score, a semi-quantitative score to evaluate the extent of the lesions, was a severity predictor in our previous study (26). However, it had no statistical correlation with the viral load. Moreover, another factor related to the extent of the lesions, e.g., number of involved lung lobe and the number of lesions were also not significantly correlated with the viral load. A low viral load may cause more serious reactions in the body, leading to a higher extent of lesions in the lung. The unexpected results may be due to the small sample size. Although the lesions were more likely be peripherally distributed and multifocal, the viral load had no predominant distributions. In other words, the distributions can be considered as a differentiated feature from other viral-related pneumonia instead of a severity predictor. Interestingly, we found that an uneasily differentiated margin indicated a lower *Ct* value, which possibly indicated the severity of the disease. The suggestion that an uneasily differentiated margin could indicate the reaction of the immune system against COVID-19 is still ongoing and the potential of further progress is expected. In contrast, an easily differentiated margin indicates that the virus has been restricted.

We also found the follow-up CT changes could help identify the patients who might progress in the later stage in our previous study (22). In this study, we also investigated the relationship between the viral load and the follow-up CT changes and found that the progressed follow-up changes were negatively correlated with the *Ct* value, which means patients with a lower *Ct* value are likely to present a progressed follow-up CT change, maybe even a worse prognosis. The *Ct* value at baseline yields an AUC of 0.685 to predict a progress follow-up CT change.

It is notable that 4 patients had no abnormal CT findings in our cohort. All 4 of the patients presented new lesions in the follow-up CT scan images, indicating that abnormal imaging findings might be absent in the early stage of COVID-19. This also further proved a lower *Ct* value are likely to present a progress follow-up CT change. It reminds physicians of the importance of follow-up CT scans for patients with normal CT findings at initial diagnosis, especially for those with a low *Ct* value.

Nevertheless, the study has several limitations. Firstly, this is the experience of a single center and the sample size was small. Our conclusions cannot be generalized to other centers taking care of COVID-19 patients directly, which needs further investigation. A multicenter study and/or including more cases might provide more information on the viral load and clinical outcomes of COVID-19. Secondly, the relationship between *Ct* value during the treatment and clinical features, laboratory tests and radiographic features were not investigated and will be conducted in a future study.

In conclusion, the viral load is negatively correlated with an uneasily differentiated lesion margin on initial CT scan images and the Ct value should be paid attention to in making the diagnosis. In addition, follow-up CT scans are necessary for patients with normal CT findings at initial diagnosis, especially for those with a low *Ct* value.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Medical Ethical Committee. Written informed consent for participation was not provided by the participants' legal guardians/next of kin because: This was a retrospective study.

AUTHOR CONTRIBUTIONS

JL, WZ, and LT: conception and design. JL and LT: administrative support. JL, WZ, and LH: provision of study materials or patients.

JL, WZ, LH, HT, and XX: collection, assembly of data, data analysis, and interpretation. All authors: manuscript writing and final approval of manuscript.

FUNDING

Funding was provided by the Key Emergency Project of Pneumonia Epidemic of novel coronavirus infection (2020SK3006), the Emergency Project of Prevention and Control for COVID-19 of Central South University (160260005), and the Foundation from Changsha Scientific and Technical Bureau, China (kq2001001). The projects who funded this study had no role in the study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

REFERENCES

1. WHO. *Novel Coronavirus – China*. (2020) Available online at: http://www.who.int/csr/don/12-january-2020-novel-coronavirus-china/en/ (accessed March 6, 2020).
2. Ren L-L, Wang YM, Wu ZQ, Xiang ZC, Guo L, Xu T, et al. Identification of a novel coronavirus causing severe pneumonia in human: a descriptive study. *Chin Med J*. (2020) 133:1015–24. doi: 10.1097/CM9.0000000000000722
3. WHO. Available online at: https://www.who.int/news-room/detail/30-01-2020-statement-on-the-second-meeting-of-the-international-health-regulations-(2005)-emergency-committee-regarding-the-outbreak-of-novel-coronavirus-(2019-ncov)
4. China National Health Commission. *Update on the Novel Coronavirus Pneumonia Outbreak*. Beijing: National Health Commission. Available online at: http://www.nhc.gov.cn/xcs/yqtb/202005/3e2bc587447f458c809f10897bb4327e.shtml (accessed May 2, 2020).
5. Drosten C, Gunther S, Preiser W, van der Werf S, Brodt HR, Becker S, et al. Identification of a novel coronavirus in patients with severe acute respiratory syndrome. *N Engl J Med*. (2003) 348:1967–76. doi: 10.1056/NEJMoa030747
6. Guan W-J, Ni Z-Y, Hu Y, Liang WH, Ou CQ, He JX, et al. Clinical characteristics of 2019 novel coronavirus infection in China. *N Engl J Med*. (2020) 382:1708–20. doi: 10.1056/NEJMoa2002032
7. Chan JFW, Yuan S, Kok KH, To KKW, Chu H, Yang J, et al. A familial cluster of pneumonia associated with the 2019 novel coronavirus indicating person-to-person transmission: a study of a family cluster. *Lancet*. (2020) 395:514–23. doi: 10.1016/S0140-6736(20)30154-9
8. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet*. (2020) 395:497–506. doi: 10.1016/S0140-6736(20)30183-5
9. Boldog P, Tekeli T, Vizi Z, Dénes A, Bartha FA, Röst G. Risk assessment of novel coronavirus 2019-nCoV outbreaks outside China. *J Clin Med*. (2020) 9:571. doi: 10.3390/jcm9020571
10. CDC. *2019 Novel Coronavirus. Prevention & Treatment. Centers for Disease Control and Prevention*. (2020). Available online at: https://www.cdc.gov/coronavirus/2019-nCoV/index.html
11. Wang FS, Zhang C. What to do next to control the 2019-nCoV epidemic? *Lancet*. (2020) 395:391–3. doi: 10.1016/S0140-6736(20)30300-7
12. Hasegawa K, Jartti T, Mansbach JM, Laham FR, Jewell AM, Espinola JA, et al. Respiratory syncytial virus genomic load and disease severity among children hospitalized with bronchiolitis: multicenter cohort studies in the United States and Finland. *J Infect Dis*. (2014) 211:1550–9. doi: 10.1093/infdis/jiu658
13. Scagnolari C, Midulla F, Selvaggi C, Monteleone K, Bonci E, Papoff P, et al. Evaluation of viral load in infants hospitalized with bronchiolitis caused by respiratory syncytial virus. *Med Microbiol Immunol*. (2012) 201:311–7. doi: 10.1007/s00430-012-0233-6
14. Liu Y, Yang Y, Zhang C, Huang F, Wang F, Yuan J, et al. Clinical and biochemical indexes from 2019-nCoV infected patients linked to viral loads and lung injury. *Sci China Life Sci*. (2020) 63:364–74. doi: 10.1007/s11427-020-1643-8
15. Zou L, Ruan F, Huang M, Liang L, Huang H, Hong Z, et al. SARS-CoV-2 viral load in upper respiratory specimens of infected patients. *N Engl J Med*. (2020) 382:1177–9. doi: 10.1056/NEJMc2001737
16. Wang D, Hu B, Hu C, Zhu F, Liu X, Zhang J, et al. Clinical characteristics of 138 hospitalized patients with 2019 novel coronavirus-infected pneumonia in Wuhan, China. *JAMA*. (2020) 323:1061–9. doi: 10.1001/jama.2020.1585
17. Chung M, Bernheim A, Mei X, Zhang N, Huang M, Zeng X, et al. CT imaging features of 2019 novel coronavirus (2019-nCoV). *Radiology*. (2020) 295:202–7. doi: 10.1148/radiol.2020200230
18. Xie X, Zhong Z, Zhao W, Zheng C, Wang F, Liu J. Chest CT for typical 2019-nCoV pneumonia: relationship to negative RT-PCR testing. *Radiology*. (2020) 296:E41–5. doi: 10.1148/radiol.2020200343
19. Pan F, Ye T, Sun P, Gui S, Liang B, Li L, et al. Time course of lung changes on chest CT during recovery from 2019 novel coronavirus (COVID-19) pneumonia. *Radiology*. (2020) 295:715–21. doi: 10.1148/radiol.2020200370
20. China National Health Commission. *Diagnosis and Treatment of Pneumonitis Caused by New Coronavirus (Trial Version 7)*. Beijing: China National Health Commission (2020). Available online at: http://www.nhc.gov.cn/yzygj/s7653p/202003/46c9294a7dfe4cef80dc7f5912eb1989.shtml (accessed June 29, 2020).
21. Laboratory diagnostics for novel coronavirus. WHO (2020). Available online at: https://www.who.int/publications/i/item/10665-330374 (accessed March 6, 2020).
22. Zhao W, Zhong Z, Xie X, Yu Q, Liu J. CT scans of patients with 2019 novel coronavirus (COVID-19) pneumonia. *Theranostics*. (2020) 10:4606–13. doi: 10.7150/thno.45016
23. Ooi GC, Khong PL, Muller NL, Yiu WC, Zhou WC, Ho JCH, et al. Severe acute respiratory syndrome: temporal lung changes at thin-section CT in 30 patients. *Radiology*. (2004) 230:836–44. doi: 10.1148/radiol.2303030853
24. Wang C, Horby PW, Hayden FG, Gao GF. A novel coronavirus outbreak of global health concern. *Lancet*. (2020) 395:470–3. doi: 10.1016/S0140-6736(20)30185-9
25. Song F, Shi N, Shan F, Zhang Z, Shen J, Lu H, et al. Emerging 2019 novel coronavirus (2019-nCoV) pneumonia. *Radiology*. (2020) 295:210–7. doi: 10.1148/radiol.2020200274
26. Zhao W, Zhong Z, Xie X, Yu Q, Liu J. Relation between chest CT findings and clinical conditions of coronavirus disease (COVID-19) pneumonia: a multicenter study. *AJR Am J Roentgenol*. (2020) 214:1072–7. doi: 10.2214/AJR.20.22976

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Public Health

ORIGINAL RESEARCH
published: 08 September 2020
doi: 10.3389/fpubh.2020.00475

Characteristic of 523 COVID-19 in Henan Province and a Death Prediction Model

Xiaoxu Ma[1†], Ang Li[2,3†], Mengfan Jiao[3†], Qingmiao Shi[3†], Xiaocai An[1†], Yonghai Feng[4], Lihua Xing[1], Hongxia Liang[3], Jiajun Chen[5], Huiling Li[1], Juan Li[3], Zhigang Ren[3], Ranran Sun[3], Guangying Cui[3], Yongjian Zhou[3], Ming Cheng[6], Pengfei Jiao[1], Yu Wang[2], Jiyuan Xing[3], Shen Shen[3], Qingxian Zhang[1], Aiguo Xu[1*] and Zujiang Yu[2,3*]*

[1] Department of Respiration, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China, [2] Department of Henan Gene Hospital, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China, [3] Department of Infectious Diseases, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China, [4] Department of Respiration, The Fifth Affiliated Hospital of Zhengzhou University, Zhengzhou, China, [5] Department of Medical Services, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China, [6] Department of Medical Information, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China

Certain high-risk factors related to the death of COVID-19 have been reported, however, there were few studies on a death prediction model. This study was conducted to delineate the clinical characteristics of patients with coronavirus disease 2019 (covid-19) of different degree and establish a death prediction model. In this multi-centered, retrospective, observational study, we enrolled 523 COVID-19 cases discharged before February 20, 2020 in Henan Province, China, compared clinical data, screened for high-risk fatal factors, built a death prediction model and validated the model in 429 mild cases, six fatal cases discharged after February 16, 2020 from Henan and 14 cases from Wuhan. Out of the 523 cases, 429 were mild, 78 severe survivors, 16 non-survivors. The non-survivors with median age 71 were older and had more comorbidities than the mild and severe survivors. Non-survivors had a relatively delay in hospitalization, with higher white blood cell count, neutrophil percentage, D-dimer, LDH, BNP, and PCT levels and lower proportion of eosinophils, lymphocytes and albumin. Discriminative models were constructed by using random forest with 16 non-survivors and 78 severe survivors. Age was the leading risk factors for poor prognosis, with AUC of 0.907 (95% CI 0.831–0.983). Mixed model constructed with combination of age, demographics, symptoms, and laboratory findings at admission had better performance ($p = 0.021$) with a generalized AUC of 0.9852 (95% CI 0.961–1). We chose 0.441 as death prediction threshold (with 0.85 sensitivity and 0.987 specificity) and validated the model in 429 mild cases, six fatal cases discharged after February 16, 2020 from Henan and 14 cases from Wuhan successfully. Mixed model can accurately predict clinical outcomes of COVID-19 patients.

Keywords: novel coronavirus pneumonia, risk factors, death prediction model, random forest, epidemiology investigation

OPEN ACCESS

Edited by:
Zisis Kozlakidis, International Agency for Research on Cancer (IARC), France

Reviewed by:
Fang Zheng, Wuhan University, China
Ana Afonso, University of São Paulo, Brazil

***Correspondence:**
Qingxian Zhang
hengzsky@aliyun.com
Aiguo Xu
aiguoxu@hotmail.com
Zujiang Yu
johnyuemzzu@163.com

[†]These authors have contributed equally to this work

Specialty section:
This article was submitted to Infectious Diseases – Surveillance, Prevention and Treatment, a section of the journal Frontiers in Public Health

Received: *15 May 2020*
Accepted: *27 July 2020*
Published: *08 September 2020*

Citation:
Ma X, Li A, Jiao M, Shi Q, An X, Feng Y, Xing L, Liang H, Chen J, Li H, Li J, Ren Z, Sun R, Cui G, Zhou Y, Cheng M, Jiao P, Wang Y, Xing J, Shen S, Zhang Q, Xu A and Yu Z (2020) Characteristic of 523 COVID-19 in Henan Province and a Death Prediction Model. Front. Public Health 8:475. doi: 10.3389/fpubh.2020.00475

INTRODUCTION

In late December 2019, Wuhan City, Hubei Province, China found several cases of unexplained pneumonia. On January 7, 2020, a new coronavirus was detected in the laboratory and the whole genome sequence of the virus was obtained. On January 12, 2020, the World Health Organization temporarily named this new virus 2019 novel coronavirus (2019-nCoV). On February 11, 2020, the World Health Organization announced that the same time the International Virus Classification Committee named the new coronavirus "SARS-CoV-2." Although the lethal rate of SARS-CoV-2 is not as high as SARS and MERS, it is more infectious than other viruses including influenza virus (1–3). The range of basic regeneration number (Ro) is estimated to be 2–5 (4, 5). China has effectively controlled the epidemic by adopting strict prevention and control measures, but in areas outside China, the epidemic of novel coronavirus is still spreading. The number of infections caused by SARS-CoV-2 is large and no specific therapeutic is available yet, which is the main cause of so many deaths. SARS-CoV-2 can cause pneumonia and systemic inflammation, leading to multiple organ failure in high-risk patients. More and more studies have focused on the high-risk factors of death. Demographic factors, advanced age, combined underlying diseases, and D-dimer exceeding 1 μg/L have been confirmed as risk factors for death in adult patients (6). In the absence of vaccines and specific antiviral drugs, targeted application of supportive therapy may be beneficial to relieve symptoms and protect organ functions (7). How to quickly identify high-risk patients in the early stage of the disease and actively adopt supportive treatment to reduce mortality is an urgent problem to be solved in the clinic. Cao Bin (6) and others reported some characteristics and clinical progress of the early stage of severe and dead patients, which improved our further understanding of the characteristics of dead patients. However, there are no relevant studies on the application of models to predict COVID-19 death. Using admission characteristics and laboratory test results to establish a predictive model can calculate the probability of over-all mortality due to SARS-CoV-2, identify high-risk patients as early as possible and give support to reduce mortality as soon as possible.

In this study, we collected data of 523 discharged cases of novel coronavirus infection in Henan Province, China and compared the demographics, clinical characteristics, laboratory test, imaging between the mild, severe survivors and non-survivors. We established a death prediction model using the data upon admission of the severe survivors and non-survivors.

METHODS

Study Design and Participants

From January 22, 2020 to February 20, 2020, a total of 717 patients confirmed COVID-19 were discharged in 18 cities of Henan Province, China, of which 19 died. We designed a data collection table, including age, gender, epidemiological history, past history, clinical symptoms, laboratory examination, chest CT and recorded the treatment process and clinical outcome, and data of 556 patients with novel coronavirus pneumonia discharged before February 20, 2020 was collected. All data were checked by two physicians (AL and XM) and a third researcher (QZ) adjudicated any difference in interpretation between the two primary reviewers. For different interpretations and missing data, we contacted the doctor who filled out the form and the patient or their family members to review and supplement. Excluding 18 cases under the age of 18, 10 cases missing key information and five cases transferred to other hospitals with no end point, 523 cases were included for statistical analysis, of which 19 cases died including three fatal cases with data missing. According to the Guidance for Corona Virus Disease 2019 (6th edition) released by the National Health Commission of China, the enrolled cases were categorized as mild or severe (8). There were no deaths in the mild. According to the clinical outcome, we divided the severe into severe survivors and non-survivors. Up to April 1, there were 22 cases died of COVID-19 in Henan Province. We have managed to collect data of another six fatal cases of Henan Province and 14 cases from the Fourth People's Hospital of Wuhan to validate the predictive power of the model. The flow diagram of included patients is shown in **Figure 1**.

Definition

The incubation period was defined as the interval between the potential earliest date of contact of the transmission source (wildlife or person of suspected or confirmed case) and the potential earliest date of symptom onset (i.e., cough, fever, fatigue, or myalgia). We excluded cases with an incubation period of <1 day or cases of continuous exposure, because those patients continued to be infected. Fever was defined as an axillary temperature of 37.3°C or higher. Lymphopenia was defined as a lymphocyte count of <1,200 per cubic millimeter. Thrombocytopenia was defined as a platelet count of <100,000 per cubic millimeter. Chest CT was divided into normal, mild, moderate and severe infections according to the range of lesions. The range of lesions < 15% was mild; the range of lesions 15–40% was moderate; the range of lesions > 40% was severe.

Statistical Analysis

Statistical analyses on cohort characteristics were performed on R version 3.6.1. Participants' demographic, laboratory findings and questionnaire were summarized with a standardized statistical significance test method, categorical variables were shown as counts and percentages [*n* (%)], and associations were tested using a fisher' exact test. Continuous variables were shown as median (interquartile range, IQR), and differences between groups were analyzed with non-parametric test (Wilcoxon's rank-sum test). A single-sided $p < 0.05$ was considered statistically significant. Discriminative models were constructed by using random forest with leave-one-out cross validation, features were selected by using embedded backward selection. Missing data were filled by chose median value in relative cohort (Severe death, severe survival, and mild) for model construction and validation. Receiver operating characteristic (ROC) curve and Precision-Recall curve were visualized by using R program package "pROC" and "precrec," respectively.

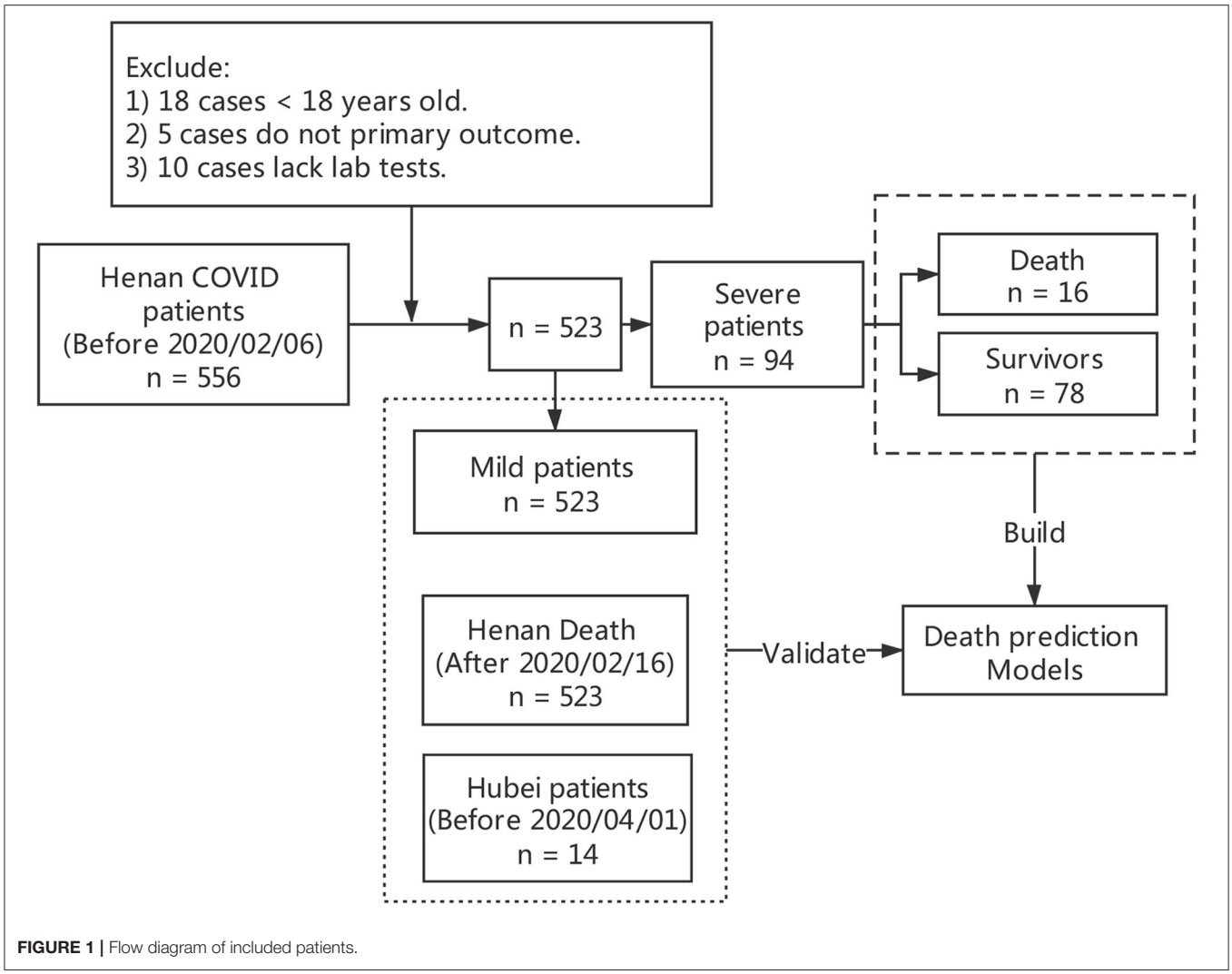


FIGURE 1 | Flow diagram of included patients.

RESULTS

Clinical Characteristics of the Study Patients According to Disease Severity and Clinical Outcome in Severe

Table 1 shows that among the 523 patients 429/523 (82.03%) were mild, 94/523 (17.97%) severe, and 16/94 (17.02%) in severe cases died of COVID-19. The median age of the 523 patients was 44.0 years (IQR 32–54), with male patients (55.26%) accounting for the majority. Severe patients were older than mild patients (50.00 [IQR 38.25–61.5] vs. 42 [IQR 31–54]), and non-survivors were older than 65 years with a median age of 71 years (IQR 67.75–80). Age difference between the cases was statistically significant.

Within 14 days before the onset, 324 (61.95%) had lived in or visited Wuhan; 158 (30.21%) had contact history with Wuhan returnees; 136 (26%) had confirmed contact history of COVID-19 cases; 76 (14.53%) occurred from familial clusters, and 67 (12.81%) had unknown contact history. Non-survivors were not much different from the mild or severe survivors in terms of epidemiological history.

Hypertension (74/465 [15.91%]), diabetes (42/462 [9.09%]), coronary heart disease (24/461 [5.21%]) were the most common comorbidities. The average number of comorbid diseases in the non-survivors was 1.94 which was significantly higher than that of the mild and severe survivors. The most common symptoms on admission were fever (449 [88.74%], cough (309 [62.3%] and fatigue (190 [39.58%]); the more common symptoms were expectoration (138 [28.75%]), chest tightness (92 [19.53%]), sore throat (65 [13.83%]), anorexia (61 [12.9%]), gasp (44 [9.4%]), and dyspnea (41 [8.7 %]). Muscle and joint pain, runny nose, diarrhea, dizziness, and headache were rare. The symptoms of fever, cough, dyspnea, gasp, chest tightness, nasal congestion, and muscle and joint pain had a higher incidence in severe cases, and the difference was significant; the incidence of chest tightness in non-survivors was higher than that in severe survivors. The patients in the non-survivors had more symptoms at the onset.

TABLE 1 | Clinical characteristics of the study patients according to disease severity and clinical outcome in severe.

Characteristics	All patients (*N* = 523)	Disease severity			Clinical outcome in severe		
		Mild (*N* = 429)	Severe (*N* = 94)	*p*-value	Non-survivors (*N* = 16)	Survivors (*N* = 78)	*p*-value
Age, years	44 (32–54)	42 (31–54)	50 (38.25–61.5)	0.00004	71 (67.75–80)	47 (34–54.75)	0
18–49	314/523 (60.04)	270/429 (62.94)	44/94 (46.81)	0.00384	1/16 (6.25)	43/78 (55.13)	0.00036
50–64	146/523 (27.92)	121/429 (28.21)	25/94 (26.6)	0.75274	1/16 (6.25)	24/78 (30.77)	0.03505
≥65	46/523 (8.8)	23/429 (5.36)	23/94 (24.47)	0	14/16 (87.5)	9/78 (11.54)	0
Female sex	234/523 (44.74)	199/429 (46.39)	35/94 (37.23)	0.06599	3/16 (18.75)	32/78 (41.03)	0.07824
Exposure to Source of Transmission Within Past 14 Days							
Had the history of travel or residence in Wuhan and its surrounding areas, or other communities where the case of COVID-19 had been reported	324/523 (61.95)	272/429 (63.4)	52/94 (55.32)	0.08999	5/16 (31.25)	47/78 (60.26)	0.03216
Had contact with Wuhan residents	158/523 (30.21)	132/429 (30.77)	26/94 (27.66)	0.32199	6/16 (37.5)	20/78 (25.64)	0.24972
Cluster	76/523 (14.53)	66/429 (15.38)	10/94 (10.64)	0.15317	2/16 (12.5)	8/78 (10.26)	0.5381
Had contact with patients confirmed0020COVID-19	136/523 (26)	115/429 (26.81)	21/94 (22.34)	0.22412	5/16 (31.25)	16/78 (20.51)	0.263
Not clear	67/523 (12.81)	48/429 (11.19)	19/94 (20.21)	0.01702	3/16 (18.75)	16/78 (20.51)	0.58924
Comorbidity							
COPD	13/463 (2.81)	6/373 (1.61)	7/90 (7.78)	0.00526	5/15 (33.33)	2/75 (2.67)	0.00117
Asthma	3/459 (0.65)	2/369 (0.54)	1/90 (1.11)	0.48126	0/15 (0)	1/75 (1.33)	0.83333
Interstitial pneumonia	8/462 (1.73)	7/372 (1.88)	1/90 (1.11)	0.51749	1/15 (6.67)	0/75 (0)	0.16667
Diabetes	42/462 (9.09)	29/371 (7.82)	13/91 (14.29)	0.0475	5/15 (33.33)	8/76 (10.53)	0.03604
Coronary heart disease	24/461 (5.21)	13/371 (3.5)	11/90 (12.22)	0.00237	5/15 (33.33)	6/75 (8)	0.01681
Hypertension	74/465 (15.91)	48/375 (12.8)	26/90 (28.89)	0.00034	7/15 (46.67)	19/75 (25.33)	0.09088
Cerebral infarction	11/461 (2.39)	9/371 (2.43)	2/90 (2.22)	0.63343	1/15 (6.67)	1/75 (1.33)	0.30712
Cerebral hemorrhage	6/459 (1.31)	4/369 (1.08)	2/90 (2.22)	0.33495	1/15 (6.67)	1/75 (1.33)	0.30712
Cancer	7/230 (3.04)	4/191 (2.09)	3/39 (7.69)	0.09676	2/10 (20)	1/29 (3.45)	0.15593
Pregnancy	2/469 (0.43)	2/382 (0.52)	0/87 (0)	0.66309	0/16 (0)	0/71 (0)	1
Digestive system disease	11/307 (3.58)	10/257 (3.89)	1/50 (2)	0.44053	0/10 (0)	1/40 (2.5)	0.8
Chronic kidney disease	5/307 (1.63)	3/256 (1.17)	2/51 (3.92)	0.19413	0/11 (0)	2/40 (5)	0.61176
Symptoms							
Fever	449/506 (88.74)	360/412 (87.38)	89/94 (94.68)	0.02648	15/16 (93.75)	74/78 (94.87)	0.6154
Fatigue	190/480 (39.58)	151/387 (39.02)	39/93 (41.94)	0.34364	10/16 (62.5)	29/77 (37.66)	0.06087
Cough	309/496 (62.3)	241/403 (59.8)	68/93 (73.12)	0.01064	11/16 (68.75)	57/77 (74.03)	0.43858
Dyspnea	41/471 (8.7)	18/378 (4.76)	23/93 (24.73)	0	7/16 (43.75)	16/77 (20.78)	0.05703
Gasp	44/468 (9.4)	22/375 (5.87)	22/93 (23.66)	0	4/16 (25)	18/77 (23.38)	0.55775
Chest tightness	92/471 (19.53)	60/378 (15.87)	32/93 (34.41)	0.0001	11/16 (68.75)	21/77 (27.27)	0.00236
Nasal congestion	32/467 (6.85)	20/374 (5.35)	12/93 (12.9)	0.01303	1/16 (6.25)	11/77 (14.29)	0.34485
Runny nose	31/467 (6.64)	21/374 (5.61)	10/93 (10.75)	0.06599	1/16 (6.25)	9/77 (11.69)	0.45525
Sore throat	65/470 (13.83)	51/378 (13.49)	14/92 (15.22)	0.38807	2/16 (12.5)	12/76 (15.79)	0.54396
Expectoration	138/480 (28.75)	107/387 (27.65)	31/93 (33.33)	0.16827	7/16 (43.75)	24/77 (31.17)	0.24499
Anorexia	61/473 (12.9)	44/380 (11.58)	17/93 (18.28)	0.06352	5/16 (31.25)	12/77 (15.58)	0.13271
Diarrhea	25/467 (5.35)	17/374 (4.55)	8/93 (8.6)	0.10097	2/16 (12.5)	6/77 (7.79)	0.41557
Headache	37/471 (7.86)	27/378 (7.14)	10/93 (10.75)	0.17119	0/16 (0)	10/77 (12.99)	0.13578
Dizziness	28/470 (5.96)	23/377 (6.1)	5/93 (5.38)	0.50991	0/16 (0)	5/77 (6.49)	0.38017
Muscle and joint pain	39/464 (8.41)	26/371 (7.01)	13/93 (13.98)	0.02981	1/16 (6.25)	12/77 (15.58)	0.29732
The Basic Vital Signs on Admission							
Respiratory rate >24 breaths per min	4/489 (0.82)	3/399 (0.75)	1/90 (1.11)	0.02936	1/13 (7.69)	0/77 (0)	0.00554
Pulse oxygen saturation <90%	8/165 (4.85)	0/122 (0)	8/43 (18.6)	0	5/11 (45.45)	3/32 (9.38)	0.00019
Fever on admission, °C	37.2 (36.7-37.9)	37.1 (36.7-37.9)	37.2 (36.8-38)	0.26312	36.7 (36.6-37.1)	37.3 (36.8-38)	0.00776
<37.5	293/496 (59.07)	237/404 (58.66)	56/92 (60.87)	0.68771	12/15 (80)	44/77 (57.14)	0.09703

(Continued)

TABLE 1 | Continued

Characteristics	All patients (*N* = 523)	Disease severity			Clinical outcome in severe		
		Mild (*N* = 429)	Severe (*N* = 94)	*p*-value	Non-survivors (*N* = 16)	Survivors (*N* = 78)	*p*-value
37.5–38.0	90/496 (18.15)	78/404 (19.31)	12/92 (13.04)	0.15947	1/15 (6.67)	11/77 (14.29)	0.37752
38.1–39.0	97/496 (19.56)	77/404 (19.06)	20/92 (21.74)	0.55865	1/15 (6.67)	19/77 (24.68)	0.10886
>39.0	16/496 (3.23)	12/404 (2.97)	4/92 (4.35)	0.34306	1/15 (6.67)	3/77 (3.9)	0.51568
Time form illness onset to seeing a doctor, days	2 (0–5)	2 (0–5)	2 (1–5)	0.41842	4.5 (1.75–7)	2 (1–4)	0.04202
Time form illness onset to hospital admission, days	4 (2–7)	4 (2–7)	3 (2–7)	0.29878	8 (6–10)	3 (1–6)	0.00047
Incubation period, days	5 (1–9)	5 (1–9)	4 (1–9)	0.30537	5 (2–10)	4 (1–8)	0.10345

Data are median (IQR)or n/N (%). P-values were calculated by Fisher's exact test or Mann-Whitney U-test. COPD, chronic obstructive pulmonary disease.

Four (0.82%) had a respiratory rate > 24 breaths/min, one of them died; 8 (4.85%) pulse oxygen saturation < 90%, all severe; median body temperature 37.2°C (IQR 36.7–37.9), 293 (59.07%) body temperature < 37.5°C, 16 (3.23%) body temperature > 39°C and 80% non-survivors body temperature < 37.5°C upon admission.

The median duration from onset of symptoms to first visit to doctor was 2 days (IQR 0–5), from onset of symptoms to first hospitalization 4 days (IQR 2–7) while 8 days (IQR 6–10) in non-survivors. The median incubation period was 5 days (IQR 1–9), with no significant difference between the cases.

Radiographic and Laboratory Findings on Admission

Table 2 shows the imaging and laboratory examination results. Of all the cases, 419 patients had detailed chest CT data on initial admission, with 17 (4.06%) being normal; 224 (53.46%) chest CT lesions < 15%; 154 (36.75%) chest CT lesions between 15 and 40%; 24 (5.73%) chest CT lesions > 40%, of which 15 were severe. In the non-survivors, 100% of patients had a chest CT lesion area of more than 15% for the first time.

In the first nucleic acid testing, 323 (65.25%) were confirmed positive for SARS-CoV-2. The leucocyte count in non-survivors (8.66×10^9/L [IQR 7–12.335]) was significantly higher than that in mild and severe survivors. Lymphocytopenia is more common in the severe than in the mild (39.24 vs. 18.16%). 96.65% of patients experienced a decrease in eosinophil count. The level of D-dimer at admission was significantly higher in severe patients (0.8 mg/L [IQR 0.19–4.18] vs. 0.39 mg/L [IQR 0.16–0.98], $p = 0.04076$), and the non-survivors was significantly higher than the survivors (6.9 mg/L [IQR 1–32.47] vs. 0.46 mg/L [IQR 0.1625–1.63225, $p = 0.00199$). The alanine aminotransferase, lactate dehydrogenase and creatine kinase in the severe were significantly higher than those in the mild, and the non-survivors was more obviously, the difference was significant. The incidence of renal impairment was higher in the non-survivors. The incidence of arterial blood gas hypoxia and respiratory alkalosis on admission in the non-survivors was higher than that in the mild and the severe survivors. Three hundred and seventy-two people were tested for C-reactive protein (CRP) upon admission. Two hundred and eleven (56.72%) had CRP > 10 mg/L. The increase rate in the severe (85.51%) was significantly higher than that in the mild (50.17%). Two hundred and thirty-five patients were tested for procalcitonin (PCT) upon admission, and 100% patients in the non-survivors had elevated PCT. Patients in non-survivors had more laboratory abnormalities than those in mild and severe.

Treatments During the Hospitalization

Two hundred and seventeen (41.49%) patients received respiratory support during hospitalization, of which 18 (4.2%) of mild patients received nasal catheter inhalation, as shown in **Table 3**. The respiratory support rate of the severe was higher than that of the mild, and the non-survivors all received mechanical ventilation treatment, of which six received non-invasive mechanical ventilation treatment and 11 received invasive mechanical ventilation treatment. Nine patients in the severe received ECMO treatment, and no one survived. Thirty-nine (52.7%) of the severe survivors were treated with CRRT, and only 5 (33.33%) of the non-survivors applied this technique. In terms of drug treatment, antiviral treatment was commonly used in each group. The severe had a higher proportion of antibiotics than the mild, and the non-survivors had a higher proportion of carbapenem and glycopeptide antibiotics than the survivors. One hundred and twelve (21.41%) received glucocorticoid therapy, and the non-survivors received a higher proportion of glucocorticoid therapy than the severe survivors (62.5 vs. 41.03%).

Death Prediction Model

We constructed classification models to evaluate death risk for severe patients. Model performance was assessed by receiver operating characteristic (ROC) curve analysis using the area under the curve (AUC). In considering age is among leading risk factors for poor prognosis in several studies (3, 6, 7, 9–11), we firstly constructed models by using single age, which could achieve and AUC of 0.907 (95% CI 0.831–0.983) for death and alive severe COVID-19 patients. Mixed models constructed with combination of age, demographics, symptoms, and laboratory tests when firstly admitted to hospital had better

TABLE 2 | Radiographic and laboratory findings on admission.

Characteristics	All patients (*N* = 523)	Disease severity			Clinical outcome in severe		
		Mild (*N* = 429)	Severe (*N* = 94)	*p*-value	Non-survivors (*N* = 16)	Survivors (*N* = 78)	*p*-value
Radiographic Findings							
Chest CT							
Normal	17/419 (4.06)	13/342 (3.8)	4/77 (5.19)	0.38245	0/10 (0)	4/67 (5.97)	0.56639
Mild	224/419 (53.46)	202/342 (59.06)	22/77 (28.57)	0	0/10 (0)	22/67 (32.84)	0.02666
Moderate	154/419 (36.75)	118/342 (34.5)	36/77 (46.75)	0.04398	6/10 (60)	30/67 (44.78)	0.2873
Severe	24/419 (5.73)	9/342 (2.63)	15/77 (19.48)	0	4/10 (40)	11/67 (16.42)	0.09699
Laboratory Findings							
Pathogens identified							
COVID-19 viral nucleic acid test positive on the first time	323/495 (65.25)	258/404 (63.86)	65/91 (71.43)	0.10514	16/16 (100)	49/75 (65.33)	0.00249
Influenza A virus Ag+	8/323 (2.48)	8/263 (3.04)	0/60 (0)	0.18936	0/7 (0)	0/53 (0)	1
Influenza B virus Ag+	13/324 (4.01)	10/264 (3.79)	3/60 (5)	0.44419	1/7 (14.29)	2/53 (3.77)	0.31543
Mycoplasma pneumonia IgM Ab+	28/319 (8.78)	22/262 (8.4)	6/57 (10.53)	0.3824	1/7 (14.29)	5/50 (10)	0.5621
HBsAg+	26/357 (7.28)	23/288 (7.99)	3/69 (4.35)	0.2214	0/11 (0)	3/58 (5.17)	0.58892
HCV-Ab+	7/353 (1.98)	7/285 (2.46)	0/68 (0)	0.22041	0/11 (0)	0/57 (0)	1
TP-Ab+	7/345 (2.03)	7/277 (2.53)	0/68 (0)	0.21186	0/11 (0)	0/57 (0)	1
Blood routine							
Leucocyte count, $\times 10^9$ /L	4.82 (3.585–6.225)	4.74 (3.5025–6.0475)	5.38 (4–7.05)	0.00281	8.66 (7–12.335)	5.12 (3.8675–5.9875)	0.00007
>10	24/487 (4.93)	14/398 (3.52)	10/89 (11.24)	0.00537	5/15 (33.33)	5/74 (6.76)	0.01073
4–10	310/487 (63.66)	252/398 (63.32)	58/89 (65.17)	0.74263	9/15 (60)	49/74 (66.22)	0.64496
<4	153/487 (31.42)	132/398 (33.17)	21/89 (23.6)	0.07869	1/15 (6.67)	20/74 (27.03)	0.07944
Platelet count, $\times 10^9$ /L	175.5 (143–210)	176 (143–209.5)	168 (145–216)	0.29403	153 (93.25–210.75)	171 (147–213.5)	0.08684
<100	26/452 (5.75)	19/375 (5.07)	7/77 (9.09)	0.13423	4/14 (28.57)	3/63 (4.76)	0.01824
Absolute lymphocyte count, $\times 10^9$ /L	1.1 (0.84–1.49)	1.12 (0.9–1.55)	0.92 (0.595–1.225)	0.00005	0.72 (0.44–0.89)	0.985 (0.6325–1.2475)	0.03876
<0.8	98/448 (21.88)	67/369 (18.16)	31/79 (39.24)	0.00004	7/13 (53.85)	24/66 (36.36)	0.23802
Lymphocyte percentage, %	24.5 (17.055–33.18)	24.8 (18.575–33.625)	19.75 (9.36–29.025)	0.00029	8.34 (5.3–13.405)	21.75 (11.225–29.775)	0.00526
<20	152/456 (33.33)	112/376 (29.79)	40/80 (50)	0.0005	11/14 (78.57)	29/66 (43.94)	0.01858
Absolute neutrophil count, $\times 10^9$ /L	3.09 (2.085–4.415)	3.04 (2.06–4.3)	3.73 (2.37–5.765)	0.01557	6.29 (4.03–11.09)	3.54 (2.25–4.62)	0.01067
Neutrophil percentage, %	65.5 (56.35–74.65)	65.4 (56.5–73.7)	68.6 (56.6–85.1)	0.00649	86.105 (80.1125–92.025)	65.95 (55.4–77.1775)	0.00172
Absolute eosinophil count, $\times 10^9$ /L	0.01 (0–0.03)	0.01 (0–0.03)	0.01 (0–0.03)	0.24402	0 (0–0.02)	0.01 (0–0.03)	0.15484
<0.2	375/388 (96.65)	314/324 (96.91)	61/64(95.31)	0.36519	10/11 (90.91)	51/53 (96.23)	0.43774
Eosinophil percentage, %	0.2 (0–0.7)	0.2 (0–0.78)	0.1 (0–0.4)	0.00506	0 (0–0.2)	0.1 (0–0.4)	0.0478
<0.1	143/405 (35.31)	109/330 (33.03)	34/75 (45.33)	0.04418	9/15 (60)	25/60 (41.67)	0.20205
<0.5	276/405 (68.15)	217/330 (65.76)	59/75 (78.67)	0.03031	13/15 (86.67)	46/60 (76.67)	0.32384
Hemagglutination examination							
Fibrinogen, g/L	3.48 (2.8–4.76)	3.43 (2.79–4.72)	3.607 (2.99–5.075)	0.18678	3.7055 (2.685–5.035)	3.607 (2.99–5.0825)	0.42719
>4	127/367 (34.6)	97/297 (32.66)	30/70 (42.86)	0.10666	5/14 (35.71)	25/56 (44.64)	0.54597
Prothrombin time, s	11.9 (10.7–12.9)	11.9 (10.77–12.9)	11.8 (10.6–13)	0.41355	12.5 (11.25–14.6)	11.7 (10.5–12.8)	0.06903
≥16	21/370 (5.68)	17/302 (5.63)	4/68 (5.88)	0.56081	1/11 (9.09)	3/57 (5.26)	0.51496
D-Dimer, mg/L	0.41 (0.18–1.77225)	0.39 (0.16–0.98)	0.8 (0.19–4.18)	0.04076	6.9 (1–32.47)	0.46 (0.1625–1.63225)	0.00199

(Continued)

TABLE 2 | Continued

Characteristics	All patients (*N* = 523)	Disease severity			Clinical outcome in severe		
		Mild (*N* = 429)	Severe (*N* = 94)	*p*-value	Non-survivors (*N* = 16)	Survivors (*N* = 78)	*p*-value
0.5–1	57/304 (18.75)	47/241 (19.5)	10/63 (15.87)	0.54445	2/13 (15.38)	8/50 (16)	0.66288
≥1	87/304 (28.62)	60/241 (24.9)	27/63 (42.86)	0.00498	10/13 (76.92)	17/50 (34)	0.00534
Liver function examination							
Glutamic-pyruvic transaminase, U/L	22.45 (15–35)	22 (15–34)	26.5 (19.375–38.825)	0.00972	28 (21–41.4)	26 (19.3–38.1)	0.38532
>40	86/378 (22.75)	69/310 (22.26)	17/68 (25)	0.62525	3/11 (27.27)	14/57 (24.56)	0.55735
Glutamic-oxalacetic transaminase, U/L	25 (19–33)	24 (18.8275–31.875)	30 (20.75–38.075)	0.00755	37 (32–42.25)	27 (20–35.4)	0.01613
>40	55/374 (14.71)	40/306 (13.07)	15/68 (22.06)	0.09858	5/11 (45.45)	10/57 (17.54)	0.05567
Total bilirubin, μmol/L	10.25 (7.575–15.35)	10.2 (7.5–14.7)	11.18 (8.05–17)	0.09421	14.755 (8.975–17.505)	10.9 (8.05–16.2)	0.16849
>17.1	61/384 (15.89)	45/311 (14.47)	16/73 (21.92)	0.11717	4/12 (33.33)	12/61 (19.67)	0.24509
Lactate dehydrogenase, U/L	208 (170.69–255.445)	202.38 (166.9–235)	246.5 (186.75–387.795)	0	437 (306.315–715.5)	223 (177.4–343.74)	0.00182
≥250	102/383 (26.63)	64/307 (20.85)	38/76 (50)	0	9/11 (81.82)	29/65 (44.62)	0.02248
Creatine Kinase, U/L	69.66 (45–113)	69 (43–106.81)	72.5 (53.99–168.3125)	0.0169	172.875 (119.75–282.84)	67.195 (53–113.75)	0.01175
≥200	36/363 (9.92)	22/299 (7.36)	14/64 (21.88)	0.00042	4/10 (40)	10/54 (18.52)	0.13805
Albumin, g/L	40.4 (36.7–44)	40.7 (37.2–44.15)	39.9 (34–42.8)	0.00594	32.8 (29.75–39.45)	39.95 (34.975–43.175)	0.01982
<30	17/380 (4.47)	8/311 (2.57)	9/69 (13.04)	0.00094	3/11 (27.27)	6/58 (10.34)	0.14825
Renal function examination							
Creatinine, μmol/L	65.29 (54–78.35)	65.29 (54–78.1)	64.85 (54.775–79.55)	0.47011	83.5 (61.38–88)	63 (54.85–71)	0.0379
≥133	5/354 (1.41)	3/284 (1.06)	2/70 (2.86)	0.25749	2/11 (18.18)	0/59 (0)	0.02277
Blood urea nitrogen, mmol/L	3.94 (3.035–5.17)	3.905 (2.9925–5.03)	4.2 (3.2–6.9625)	0.08176	5.695 (4.2075–9.99)	3.815 (3.175–5.5725)	0.07124
>8	49/332 (14.76)	35/266 (13.16)	14/66 (21.21)	0.09869	4/10 (40)	10/56 (17.86)	0.12549
Glomerular filtration rate, ml/min/1.73 m²	106.39 (89.12–120.9885)	106.49 (88.239–120.9885)	105.0085 (89.6875–121.645)	0.48903	114 (106.59–122.84)	104.17 (88.48–118.3)	0.35179
>120	16/55 (29.09)	12/39 (30.77)	4/16 (25)	0.46779	1/3 (33.33)	3/13 (23.08)	0.60714
Arterial blood gas analysis							
PH value	7.435 (7.41–7.47)	7.43 (7.409–7.46)	7.45 (7.42625–7.48)	0.02645	7.4475 (7.425–7.4675)	7.455 (7.4285–7.48)	0.29188
>7.45	41/97 (42.27)	21/61 (34.43)	20/36 (55.56)	0.04183	5/10 (50)	15/26 (57.69)	0.48092
7.35–7.45	54/97 (55.67)	39/61 (63.93)	15/36 (41.67)	003294	4/10 (40)	11/26 (42.31)	0.60234
<7.35	2/97 (2.06)	1/61 (1.64)	1/36 (2.78)	0.60696	1/10 (10)	0/26 (0)	0.27778
PCO_2, mmHg	35.7 (31.7–39)	36.3 (32–39.2)	35 (30.775–38.45)	0.0975	32.5 (26.4–35.55)	35.05 (31.85–38.55)	0.10487
<35	40/97 (41.24)	23/61 (37.7)	17/36 (47.22)	0.35762	7/10 (70)	10/26 (38.46)	0.09239
35–45	51/97 (52.58)	34/61 (55.74)	17/36 (47.22)	0.41712	2/10 (20)	15/26 (57.69)	0.04698
>45	6/97 (6.19)	4/61 (6.56)	2/36 (5.56)	0.60563	1/10 (10)	1/26 (3.85)	0.48413
PO_2, mmHg	80.8 (67–96.6)	86.9 (78.8–103)	68.5 (53.8–83.65)	0.00008	62.6 (49.275–86.45)	68.5 (58.575–81.5)	0.2567
<60	17/97 (17.53)	5/61 (8.2)	12/36 (33.33)	0.00166	5/10 (50)	7/26 (26.92)	0.17793
Other examination							
Brain natriuretic peptide, pg/mL	117.8 (53.5625–604.9)	90.71 (41–109)	612.5 (256–1121)	0	455.95 (314.715–666.75)	736.4 (256–1166)	0.22236
>100	46/74 (62.16)	17/41 (41.46)	29/33 (87.88)	0.00004	8/8 (100)	21/25 (84)	0.30914

(Continued)

TABLE 2 | Continued

Characteristics	All patients (*N* = 523)	Disease severity			Clinical outcome in severe		
		Mild (*N* = 429)	Severe (*N* = 94)	*p*-value	Non-survivors (*N* = 16)	Survivors (*N* = 78)	*p*-value
C-reactive protein, mg/L	11.425 (4.075–28)	10 (3.45–22.715)	30.86 (11–73.49)	0	68 (30.91–96)	21.105 (10.6175–50.175)	0.02518
>10	211/372 (56.72)	152/303 (50.17)	59/69 (85.51)	0	12/13 (92.31)	47/56 (83.93)	0.39436
Procalcitonin, ng/mL	0.07 (0.05–0.1525)	0.055 (0.05–0.11)	0.13 (0.05925–0.20475)	0.00012	0.1835 (0.15625–0.3225)	0.12 (0.05225–0.20475)	0.04056
<0.1	132/235 (56.17)	116/187 (62.03)	16/48 (33.33)	0.00035	0/8 (0)	16/40 (40)	0.02787
0.1–0.25	63/235 (26.81)	41/187 (21.93)	22/48 (45.83)	0.00085	6/8 (75)	16/40 (40)	0.07686
0.25–0.5	25/235 (10.64)	20/187 (10.7)	5/48 (10.42)	0.95548	0/8 (0)	5/40 (12.5)	0.38428
≥0.5	15/235 (6.38)	10/187 (5.35)	5/48 (10.42)	0.16861	2/8 (25)	3/40 (7.5)	0.18874
Cardiac troponin T, μg/L	0.01 (0.004–0.0895)	0.01 (0.003–0.2)	0.0135 (0.0075–0.04525)	0.37652	0.015 (0.012–0.079)	0.01 (0.007–0.031)	0.1928
>0.2	11/47 (23.4)	9/33 (27.27)	2/14 (14.29)	0.28702	1/5 (20)	1/9 (11.11)	0.6044
Cardiac troponin I, μg/L	0.24 (0.055–0.4775)	0.24 (0.1–0.4)	0.29 (0.02–0.9)	0.38233	0.045 (0.014–0.6925)	0.4 (0.25–5.25)	0.2669
>1.5	6/42 (14.29)	3/29 (10.34)	3/13 (23.08)	0.262	1/6 (16.67)	2/7 (28.57)	0.56294

Data are median (IQR)or n/N (%). P-values were calculated by Fisher's exact test or Mann-Whitney U-test. CT, computerized tomography; Ag, antigen; IgM, immunoglobulin m; Ab, antibody; HBsAg, hepatitis B surface antigen; HCV-Ab, hepatitis C virus antibody; TP-Ab, treponema pallidum antibody; PH, pondus hydrogenii; PCO_2, partial pressure of carbon dioxide; PO_2, partial pressure of oxygen.

performance ($p = 0.021$) and could achieved an AUC of 0.984 (95% CI 0.961–1) for death and alive severe COVID patients (**Figures 2A,B**). In considering fetal cases are with a small sample size, we randomly chose 40 samples from severe cases, then calculated the generalized AUC by using death probabilities and the median generalized AUC was 0.9852 (**Figure 2C**). Pulse oxygen, age, creatinine, creatine kinase, D-Dimer are the most important features (**Table 4**). We chose 0.441 as death prediction threshold (with 0.85 sensitivity and 0.987 specificity), then used six additional fatal cases (Henan), 429 mild cases and 14 cases (Wuhan) as independent validation cohort, and four in six death cases (0.67%) were assigned as death and majority of predicted death probabilities in the mild Henan cases and those Wuhan cases were below 0.441 (**Figure 2D**). Summary characteristics of six Henan additional fatal cases and 14 Wuhan cases and were outlined in **Table 5**.

DISCUSSION

Henan Province has a large population of 95.593 million people, bordering Hubei Province, China. As of April 1, 2020, there were 1,273 people confirmed COVID-19 in Henan, which was the second most in China outside Hubei Province. We collected data of 523 confirmed COVID-19 cases who had been discharged from 18 cities in Henan Province before February 20, 2020 and conducted statistical analysis. Our data showed that the main epidemiological characteristics of novel coronavirus pneumonia in Henan Province were import and cluster, which were similar to other provinces and cities outside Hubei in China. Among the 523 cases, there were 289 males (55.26%) and 234 females (44.74%). Other reports also showed a higher percentage of males (9, 12, 13), suggesting that males were more susceptible. Our study suggested that people of all ages were generally susceptible, with people aged 18–64 accounting for 87.96%, which was consistent with the Chinese CDC report (3). In our study, there were 16 fatal cases before February 20, 2020, and 87.5% of the deaths were ≥65 years old, with a median age of 71 years, while the median age for the mild and severe survivors was 42 and 50 years, respectively. The most common comorbidities in the non-survivors were hypertension (46.67%), coronary heart disease (33.33%), diabetes (33.33%), and COPD (33.33%). The average number of comorbidities in non-survivors was 1.94. Several studies about severe novel coronavirus pneumonia in China suggested that advanced age and comorbidities were high-risk factors for COVID-19 patients to develop into severe and death (10, 13, 14). In our study, advanced age was the biggest risk factor for death, which was consistent with that. A study from Italy involving 1,043 critically ill COVID-19 cases showed similar results, but male patients accounted for a higher proportion (82%) (9).

The median incubation period of the 523 cases in Henan Province was 5 days, and there was no significant difference between mild and severe. The median time from the onset of symptoms to hospitalization in the non-survivors was 8 days, and it was significantly longer than the severe survivors, suggesting that a delay in hospitalization might be one of the factors leading to death. Fever (88.74%), cough (62.3%), fatigue (39.58%), and expectoration (28.75%) were the most common symptoms. In spite of more symptoms, 60.87% of the severe and 80% of non-survivors had a temperature below 37.5°C at the time of admission. Zhong et al.'s study on 1,099 cases of COVID-19 also found that 52% of patients did not have

TABLE 3 | Treatments during the hospitalization.

Treatments	All patients (*N* = 523)	Disease severity		Clinical outcome in severe	
		Mild (*N* = 429)	Severe (*N* = 94)	Non-survivors (*N* = 16)	Survivors (*N* = 78)
Oxygen support	217/523 (41.49)	152/429 (35.43)	65/94 (69.15)	15/16 (93.75)	50/78 (64.1)
Oxygen inhalation through nasal catheter	73/523 (13.96)	18/429 (4.2)	55/94 (58.51)	9/16 (56.25)	46/78 (58.97)
Usage time, days	10 (6–14.5)	10 (7–14)	7.5 (4–14.75)	3.5 (2–4.25)	11 (4–15.75)
High-flow oxygen	48/523 (9.18)	0/429 (0)	48/94 (51.06)	10/16 (62.5)	38/78 (48.72)
Usage time, days	6 (4–11)	NA (NA-NA)	6 (4–11)	3.5 (2–4.75)	8 (4.75–12.25)
Non-invasive mechanical ventilation	15/523 (2.87)	0/429 (0)	15/94 (15.96)	6/16 (37.5)	9/78 (11.54)
Usage time, days	6 (5–12)	NA (NA-NA)	6 (5–12)	4.5 (3.25–7.25)	7 (6–13)
Invasive mechanical ventilation	13/523 (2.49)	0/429 (0)	13/94 (13.83)	11/16 (68.75)	2/78 (2.56)
Usage time, days	2 (1.25–7.75)	NA (NA-NA)	2 (1.25–7.75)	2 (1–4)	13 (13–13)
ECMO	9/523 (1.72)	0/429 (0)	9/94 (9.57)	9/16 (56.25)	0/78 (0)
Usage time, days	7 (2–9)	NA (NA-NA)	7 (2–9)	7 (2–9)	NA (NA-NA)
CRRT	44/449 (9.8)	0/360 (0)	44/89 (49.44)	5/15 (33.33)	39/74 (52.7)
Adsorptive	12/44 (27.27)	0/0 (NA)	12/44 (27.27)	3/5 (60)	9/39 (23.08)
Usage time, times	3 (2–3)	NA (NA-NA)	3 (2–3)	2 (1.5–2.5)	3 (3–3)
Non-adsorptive	32/44 (72.73)	0/0 (NA)	32/44 (72.73)	2/5 (40)	30/39 (76.92)
Usage time, times	2 (2–2)	NA (NA-NA)	2 (2–2)	NA (NA-NA)	2 (2–2)
Drug Treatment	497/523 (95.03)	408/429 (95.1)	89/94 (94.68)	14/16 (87.5)	75/78 (96.15)
Antiviral treatment					
Other	174/523 (33.27)	142/429 (33.1)	32/94 (34.04)	4/16 (25)	28/78 (35.9)
Immunotherapy	181/523 (34.61)	126/429 (29.37)	55/94 (58.51)	13/16 (81.25)	42/78 (53.85)
Methylprednisolone	103/523 (19.69)	61/429 (14.22)	42/94 (44.68)	10/16 (62.5)	32/78 (41.03)
Usage time, days	5 (3–6)	5 (3–6)	5 (3–6)	5 (3–6)	5 (3–6)
Prednisone	9/523 (1.72)	9/429 (2.1)	0/94 (0)	0/16 (0)	0/78 (0)
Usage time, days	6 (4–14)	6 (4–14)	NA (NA-NA)	NA (NA-NA)	NA (NA-NA)
Immunoglobulin	80/523 (15.3)	43/429 (10.02)	37/94 (39.36)	12/16 (75)	25/78 (32.05)
Usage time, days	4 (3–5)	4 (3–4.25)	4 (2–5)	2.5 (1.75–4.25)	4 (3–5)
Thymosin α	68/523 (13)	50/429 (11.66)	18/94 (19.15)	4/16 (25)	14/78 (17.95)
Usage time, days	8.5 (6–13.75)	8 (5–13)	11 (8–15)	9 (6.5–10)	11 (8.25–15.75)
Antibiotics	347/523 (66.35)	279/429 (65.03)	68/94 (72.34)	12/16 (75)	56/78 (71.79)
Quinolone	266/523 (50.86)	220/429 (51.28)	46/94 (48.94)	5/16 (31.25)	41/78 (52.56)
Penicillin	67/523 (12.81)	51/429 (11.89)	16/94 (17.02)	3/16 (18.75)	13/78 (16.67)
Cephems	99/523 (18.93)	72/429 (16.78)	27/94 (28.72)	6/16 (37.5)	21/78 (26.92)
Carbapenem	25/523 (4.78)	11/429 (2.56)	14/94 (14.89)	6/16 (37.5)	8/78 (10.26)
Glycopeptide	9/523 (1.72)	2/429 (0.47)	7/94 (7.45)	5/16 (31.25)	2/78 (2.56)
Tetracycline	11/523 (2.1)	3/429 (0.7)	8/94 (8.51)	2/16 (12.5)	6/78 (7.69)
Antifungai agents	26/523 (4.97)	6/429 (1.4)	20/94 (21.28)	10/16 (62.5)	10/78 (12.82)
Fluconazole	4/523 (0.76)	0/429 (0)	4/94 (4.26)	2/16 (12.5)	2/78 (2.56)
Voriconazole	11/523 (2.1)	1/429 (0.23)	10/94 (10.64)	6/16 (37.5)	4/78 (5.13)
Caspofungin	8/523 (1.53)	0/429 (0)	8/94 (8.51)	5/16 (31.25)	3/78 (3.85)
Anti-inflammatory treatment	198/523 (37.86)	148/429 (34.5)	50/94 (53.19)	11/16 (68.75)	39/78 (50)
Acetylcysteine Effervescent Tablets	38/523 (7.27)	27/429 (6.29)	11/94 (11.7)	1/16 (6.25)	10/78 (12.82)

Data are median (IQR)or n/N (%). ECMO, extracorporeal membrane oxygenation; CRRT, continuous renal replacement therapy.

fever when they became ill (12). The lack of fever symptoms made it difficult to identify COVID-19 patients and could also be one of the factors that caused a delay in visiting the doctor. Another study on refractory COVID-19 also found that the refractory pneumonia cases had a significantly lower fever incidence than the common pneumonia cases, suggesting that slow or poor response to SARS-CoV-2 was more likely to cause severe illness (15).

Compared with the mild and severe survivors, the non-survivors had higher leucocyte count, neutrophil percentage, D-dimer, LDH, BNP, and PCT levels, while the proportion of eosinophils, lymphocytes and albumin were lower, which

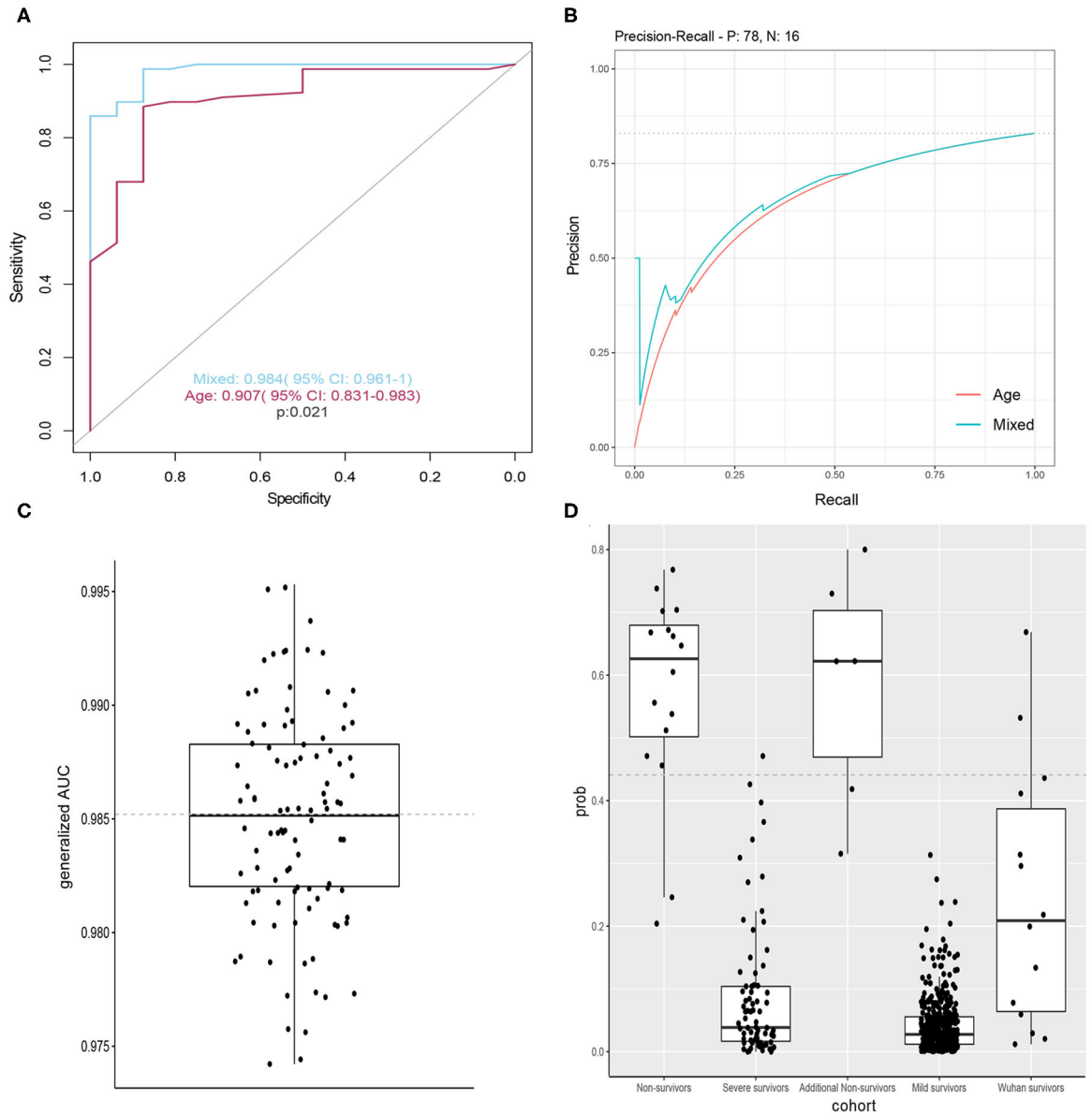


FIGURE 2 | Models to predict death risk. **(A)** Performance of the classifiers using AUCs, significance determined by single sided AUC comparison by using bootstrap method with 10,000 permutations (boot. $n = 10{,}000$). **(B)** Precision-recall curves of models based on mixed features and age. **(C)** Distribution of generalized AUC by using bootstrap sampling ($n = 100$). **(D)** Boxplots showing distribution of death probabilities among different cohorts. horizon dashed line indicates selected threshold.

was consistent with other studies. White blood cell count, neutrophil percentage and elevated PCT suggested that the non-survivors might be hospitalized with bacterial infection. Low albumin indicated that the patient was seriously depleted and the nutritional level was poor. D-dimer elevation had been confirmed in multiple studies as a high-risk factor for severe illness and death (10, 16, 17), which was consistent with our study. Chen et al.'s study found that in the non-survivors 56% had increased leucocyte count and 91% had lymphopenia, while in the severe survivors 4% had increased leucocyte count and 47% had lymphopenia (10). Zhang et al.'s study found that most COVID-19 cases combined with lymphopenia (75.4%) and eosinophilia (52.9%), and lymphopenia and eosinophilia were associated with disease severity (17). In our study, eosinophilia generally occurred in all cases, and there was no significant difference between the non-survivors and the severe survivors, but most of the eosinophils in the severe survivors returned to normal when discharged, while that of the non-survivors continued to decrease. Liu et al. also found that eosinophilia might be an indicator of disease improvement (18).

In the non-survivors, 100% of the patients had chest CT pneumonia area > 15% at admission, which was more severe

TABLE 4 | Importance of features in death risk prediction model.

Feature	Mean decrease Gini
Pulse oxygen saturation <90%	3.234
Age	3.025
Creatinine	1.907
Creatine Kinase	1.903
D-Dimer	1.787
Neutrophil percentage	1.292
Lactic dehydrogenase	1.274
Leucocyte count	1.207
Albumin	1.047
Time form illness onset to hospital admission	0.815
Glutamic-oxalacetic transaminase	0.737
Neutrophil count	0.731
Lymphocyte percentage	0.685
Respiratory rate >24 breaths per min	0.613
Prothrombin time	0.587
Blood urea nitrogen	0.562
Platelet	0.504
Direct bilirubin	0.486
C-reactive protein	0.405
Incubation period	0.388
Eosinophil percentage	0.264
Temperature	0.259
Chronic respiratory disease	0.207
Chronic obstructive pulmonary disease	0.202
Chest tightness	0.125
Diabetes	0.119
Coronary heart disease	0.116
Chest CT	0.095
Cardiovascular disease	0.072
Hypertension	0.064
Dyspnea	0.057
Cluster	0.050
Expectoration	0.031

CT, computerized tomography.

TABLE 5 | Summary characteristics of six Henan additional fatal cases and 14 Wuhan cases.

Feature	WH (n=14)	HN (N=6)
Age	63.5 (45–75.5)	77 (65–78.5)
Time form illness onset to hospital admission	10 (7–14.75)	2.5 (1–7)
Breath	21.5 (20.25–22.75)	24.5 (23.25–25.75)
Cardiovascular disease	3/14 (21.43)	4/5 (80)
Diabetes	2/14 (14.29)	1/5 (20)
Coronary heart disease	2/14 (14.29)	1/5 (20)
Systolic pressure	128 (123.25–133)	157.5 (144.5–160)
Chest tightness	10/14 (71.43)	2/6 (33.33)
Pulse oxygen saturation	92.5 (90–94.75)	93.5 (89–95)
Chronic respiratory disease	1/14 (7.14)	2/5 (40)
Chronic obstructive pulmonary disease	1/14 (7.14)	2/5 (40)
Hypertension	5/14 (35.71)	4/5 (80)
Temperature	36.75 (36.5–36.88)	37 (36.58–37.43)
Sputum	2/14 (14.29)	4/6 (66.67)
Cluster	2/14 (14.29)	1/6 (16.67)
Dyspnea	7/14 (50.00)	1/6 (16.67)
Incubation period	0 (0–0)	8 (4–10.5)
White Blood Cell	5.95 (4.74–7.09)	8.04 (6.52–10.53)
Aspartate Aminotransferase	25 (23–30)	28.5 (18.5–50.5)
Lactic dehydrogenase	175.5 (153.5–235)	566.5 (294.25–876.25)
Blood urea nitrogen	6.13 (4.32–7.27)	10.46 (5.01–18.28)
Neutrophil percentage	69.4 (62.7–79.1)	92.35 (90.83–94.4)
Albumin	32.8 (30.2–37.6)	33.2 (29.38–35.75)
Creatinine	70 (60.2–103)	57 (42.36–67.25)
D-Dimer	0.71 (0.33–0.86)	40.31 (40.31–40.31)
Neutrophil count	4.33 (3.23–5.29)	7.33 (6.1–9.92)
Creatine Kinase	66.5 (50.25–115.25)	133.5 (66.25–197.75)
Platelet	235.5 (196.25–309)	110 (98–156.5)
Direct bilirubin	4.2 (3.1–6.1)	4.1 (3.1–13.5)
Lymphocyte percentage	18.6 (12.8–28)	5 (3.33–6.3)
Eosinophil percentage	0.6 (0–1)	0.1 (0.03–0.33)
C-reactive protein	23.35 (3.95–66.53)	55.25 (19.74–106.43)
Prothrombin time	11.5 (10.7–12.2)	14.2 (14.1–14.3)

Data are median (IQR)or n/N (%).

in imaging than the mild and severe survivors. In terms of respiratory support, the rate of mechanical ventilation in the non-survivors was significantly higher than that in the mild and the severe survivors, which also suggested that the lung function of the non-survivors was more seriously impaired. In the non-survivors, the percentage of invasive mechanical ventilation was 68.75%, higher than other reports from Wuhan, China, but lower than those reported by the United States (71%) and Italy (88%), and Henan Province's mortality rate was also lower than that of the United States and Italy (9, 19). In addition to the aging factor, the fatal rate difference between Italy and Henan Province could be due to the fact that the number of COVID-19 cases in Henan province was relatively smaller and the medical resources were relatively more sufficient. Nine patients were applied with extracorporeal membrane oxygenation and technology (ECMO), but no one survived. Research showed application of ECMO could reduce mortality of patients with H1N1-related ARDS and MERS-related ARDS (20, 21), but there was no large-scale clinical report on the application of ECMO in the treatment of COVID-19, and its success rate is still unclear. In Yang et al.'s report, six patients applied ECMO with only one survived (13). Only a few cases were reported with successful ECMO treatment (22, 23). The recovery of lymphocyte count was the key factor for improvement of COVID-19. The application of ECMO destroyed lymphocytes and affected the function of lymphocytes. At the same time, it could cause IL-6 increase. This could be a reason for the low success rate of ECMO treatment. How to successfully apply ECMO in the treatment of COVID-19 still requires further research. 52.7% of patients in the critical severe survivors applied CRRT technology, while 33.33% patients in the non-survivors, suggesting that CRRT could help improve the prognosis of COVID-19. The application rate of glucocorticoids in the non-survivors was significantly higher than that in the mild and the severe survivors, which was consistent with other studies.

Glucocorticoids had been widely used in SARS-CoV and MERS-CoV, but studies showed that the application of glucocorticoids prolonged the clearance time of virus and the probability of mental illness was significantly increased (24). Similarly, there was no evidence that glucocorticoids were beneficial to improve the prognosis of patients with COVID-19. Whether glucocorticoids can improve the prognosis of COVID-19 still requires long-term follow-up and further research.

In our study, some independent risk factors for death were found and we firstly developed a forest tree to accurately predict clinical outcomes of patients with COVID-19 based on combination of age, demographic features, symptoms and clinical tests at admission. Old age was the most important risk factor for poor prognosis of COVID-19 patients. The mixed model conducted by forest tree performed well in predicting survival and death, with AUC of 0.984 (95% confidence interval 0.961–1) for survival and death, which is helpful for further understanding and improve clinical strategies against COVID-19. We also found the predicted value was positively correlated with the severity of COVID-19. Of the 14 confirmed cases from Wuhan, seven were mild, seven were severe, 13 were cured and discharged, and one was referred to other hospital due to critical illness. In the death prediction model based on Wuhan data, those with a predicted value >0.3 were all critically ill, and the respiratory support treatment intensity was higher than the other 10 cases. The predictive value of the case transferred to other hospitals due to critical illness was 0.673, unfortunately we failed to follow up on the clinical outcome. The death prediction model we have established has also been validated in mild and six other fatal cases in Henan Province. The prediction of death for all mild survivors was below 0.3 and 4 in six death cases (66.67%) were assigned as death.

Mild patients have rare fetal cases thus we excluded mild cases in the death prediction models. Several studies have constructed models for early identification of cases at high risk of progression to severe COVID-19 (11) or improved prognosis (25). However, fatal cases were always rapid disease progression and died in hospitals in a short time, though we have plenty of medical support in Henan province. To the best of our knowledge, this is the first death prediction model for COVID-19 established by random forest. The model can accurately predict the prognosis of patients with COVID-19. Our study provided a new method for the evaluation of disease severity. Early identification of high-risk COVID-19 cases and early supportive therapy is critical to the prognosis.

There are some limitations of our study. Firstly, this is a retrospective study. There was incomplete documentation of the history, symptoms, or laboratory findings in some cases, even after trying to feedback and recollect. Secondly, as a retrospective and observational study, although this random forest model was validated in mild cases and additional fatal cases in Henan Province and 14 cases from Wuhan and showed good predictive effects, there were few validators outside Henan Province. Thirdly, imageology lacked objective judgment standards, and the investigators' judgment was subjective, which might lead to some bias.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation. The datasets generated for this study can be found here: https://github.com/xiaoshubaba/COVID-Henan.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee from The First Affiliated Hospital of Zhengzhou University. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

QZ, AX, and ZY made substantial contributions to conception, designed the study, had full access to all of the data in the study, take responsibility for the integrity of the data, and the accuracy of the data analysis. XM and AL drafted the manuscript, critically revised the manuscript for important intellectual content, and gave final approval for the version to be published. XM, AL, MJ, QS, and XA did the data analysis. XM, AL, MJ, QS, XA, YF, HLia, JC, HLi, JL, ZR, RS, GC, YZ, MC, LX, PJ, and YW collected the data and checked the data. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

FUNDING

This work was supported by Chinese National Science and Technology Major Project (2018ZX10305410); Henan Province Medical Science and Technique Project (2018020001); Henan Province postdoctoral research grant (001801005); Henan Province Key Scientific Research Projects of Higher Education Institutions (20B320032); and Beijing Medical and Health Public Welfare Foundation (YWJKJJHKYJJ-B182121-1).

ACKNOWLEDGMENTS

The authors are very grateful to all the investigators who collected the clinical data of this study.

REFERENCES

1. Meo SA, Alhowikan AM, Al-Khlaiwi T, Meo IM, Halepoto DM, Iqbal M, et al. Novel coronavirus 2019-nCoV: prevalence, biological and clinical characteristics comparison with SARS-CoV and MERS-CoV. *Eur Rev Med Pharmacol Sci.* (2020) 24:2012–9. doi: 10.26355/eurrev_202002_20379
2. Zumla A, Chan JF, Azhar EI, Hui DS, Yuen KY. Coronaviruses—drug discovery and therapeutic options.

Nat Rev Drug Discov. (2016) 15:327–47. doi: 10.1038/nrd.2015.37
3. Wu Z, McGoogan JM. Characteristics of and important lessons from the coronavirus disease 2019 (COVID-19) outbreak in China: summary of a report of 72,314 cases from the Chinese Center for Disease Control and Prevention. *JAMA.* (2020) 323:1239–42. doi: 10.1001/jama.2020.2648
4. Zhao S, Lin Q, Ran J, Musa SS, Yang G, Wang W, et al. Preliminary estimation of the basic reproduction number of novel coronavirus (2019-nCoV) in China, from 2019 to 2020: a data-driven analysis in the early phase of the outbreak. *Int J Infect Dis.* (2020) 92:214–7. doi: 10.1101/2020.01.23.916395
5. Chen J. Pathogenicity and transmissibility of 2019-nCoV-A quick overview and comparison with other emerging viruses. *Microbes Infect.* (2020) 22:69–71. doi: 10.1016/j.micinf.2020.01.004
6. Zhou F, Yu T, Du R, Fan G, Liu Y, Liu Z, et al. Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: a retrospective cohort study. *Lancet.* (2020) 395:1054–62. doi: 10.1016/S0140-6736(20)30566-3
7. Weiss P, Murdoch DR. Clinical course and mortality risk of severe COVID-19. *Lancet.* (2020) 395:1014–5. doi: 10.1016/S0140-6736(20)30633-4
8. National Health Commission of the Pople's Republic of China. *New Coronavirus Pneumonia Prevention and Control Program.* 6th ed. National Health Commission of the Pople's Republic of China. (2020) Available online at: http://www.nhc.gov.cn/yzygj/s7653p/202002/8334a8326dd94d329df351d7da8aefc2/files/b218cfeb1bc54639af227f922bf6b817 (accessed May 7, 2020).
9. Grasselli G, Zangrillo A, Zanella A, Antonelli M, Cabrini L, Castelli A, et al. Baseline characteristics and outcomes of 1,591 patients infected with SARS-CoV-2 admitted to ICUs of the Lombardy Region, Italy. *JAMA.* (2020) 323:1574–81. doi: 10.1001/jama.2020.5394
10. Chen T, Wu D, Chen H, Yan W, Yang D, Chen G, et al. Clinical characteristics of 113 deceased patients with coronavirus disease 2019: retrospective study. *BMJ.* (2020) 368:m1091. doi: 10.1136/bmj.m1091
11. Gong J, Ou J, Qiu X, Jie Y, Chen Y, Yuan L, et al. A tool to early predict severe corona virus disease 2019 (COVID-19): a multicenter study using the risk nomogram in Wuhan and Guangdong, China. *Clin Infect Dis Off Publ Infect Dis Soc Am.* (2020) 71:833–40. doi: 10.1093/cid/ciaa443
12. Guan WJ, Ni ZY, Hu Y, Liang WH, Ou CQ, He JX, et al. Clinical characteristics of coronavirus disease 2019 in China. *N Engl J Med.* (2020) 382:1708–20. doi: 10.1056/NEJMoa2002032
13. Yang X, Yu Y, Xu J, Shu H, Xia J, Liu H, et al. Clinical course and outcomes of critically ill patients with SARS-CoV-2 pneumonia in Wuhan, China: a single-centered, retrospective, observational study. *Lancet Respir Med.* (2020) 8:475–81. doi: 10.1016/S2213-2600(20)30079-5
14. Wu C, Chen X, Cai Y, Xia J, Zhou X, Xu S, et al. Risk factors associated with acute respiratory distress syndrome and death in patients with coronavirus disease 2019 pneumonia in Wuhan, China. *JAMA Intern Med.* (2020) 180:1–11. doi: 10.1001/jamainternmed.2020.0994
15. Mo P, Xing Y, Xiao Y, Deng L, Zhao Q, Wang H, et al. Clinical characteristics of refractory COVID-19 pneumonia in Wuhan, China. *Clin Infect Dis.* (2020) 58:ciaa270. doi: 10.1093/cid/ciaa270
16. Han H, Yang L, Liu R, Liu F, Wu KL, Li J, et al. Prominent changes in blood coagulation of patients with SARS-CoV-2 infection. *Clin Chem Lab Med.* (2020) 58:1116–20. doi: 10.1515/cclm-2020-0188
17. Zhang JJ, Dong X, Cao YY, Yuan YD, Yang YB, Yan YQ, et al. Clinical characteristics of 140 patients infected with SARS-CoV-2 in Wuhan, China. *Allergy.* (2020) 75:1730–41. doi: 10.1111/all.14238
18. Liu F, Xu A, Zhang Y, Xuan W, Yan T, Pan K, et al. Patients of COVID-19 may benefit from sustained lopinavir-combined regimen and the increase of eosinophil may predict the outcome of COVID-19 progression. *Int J Infect Dis.* (2020) 95:183–91. doi: 10.1016/j.ijid.2020.03.013
19. Arentz M, Yim E, Klaff L, Lokhandwala S, Riedo FX, Chong M, et al. Characteristics and outcomes of 21 critically ill patients with COVID-19 in Washington State. *JAMA.* (2020) 323:1612–4. doi: 10.1001/jama.2020.4326
20. Noah MA, Peek GJ, Finney SJ, Griffiths MJ, Harrison DA, Grieve R, et al. Referral to an extracorporeal membrane oxygenation center and mortality among patients with severe 2009 influenza A(H1N1). *JAMA.* (2011) 306:1659–68. doi: 10.1001/jama.2011.1471
21. Hong X, Xiong J, Feng Z, Shi Y. Extracorporeal membrane oxygenation (ECMO): does it have a role in the treatment of severe COVID-19. *Int J Infect Dis.* (2020) 94:78–80. doi: 10.1016/j.ijid.2020.03.058
22. Zhan WQ, Li MD, Xu M, Lu YB. Successful treatment of COVID-19 using extracorporeal membrane oxygenation, a case report. *Eur Rev Med Pharmacol Sci.* (2020) 24:3385–9. doi: 10.26355/eurrev_202003_20705
23. Tavazzi G, Pellegrini C, Maurelli M, Belliato M, Sciutti F, Bottazzi A, et al. Myocardial localization of coronavirus in COVID-19 cardiogenic shock. *Eur J Heart Fail.* (2020) 22:911–5. doi: 10.1002/ejhf.1828
24. Russell CD, Millar JE, Baillie JK. Clinical evidence does not support corticosteroid treatment for 2019-nCoV lung injury. *Lancet.* (2020) 395:473–5. doi: 10.1016/S0140-6736(20)30317-2
25. Jiaojiao Xie, Ding Shi, Mingyang Bao, Xiaoyi Hu, Wenrui Wu, Jifang Sheng, et al. A predictive nomogram for predicting improved clinical outcome probability in patients with COVID-19 in Zhejiang Province, China. *Engineering.* (2020). doi: 10.1016/j.eng.2020.05.014. [Epub ahead of print].

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Medicine

ORIGINAL RESEARCH
published: 09 September 2020
doi: 10.3389/fmed.2020.567199

The Impact of SARS-CoV-2 on the Most Common Comorbidities–A Retrospective Study on 814 COVID-19 Deaths in Romania

Madalina Gabriela Barbu[1†], *Richard James Thompson*[2†], *Dana Claudia Thompson*[1*], *Dragos Cretoiu*[1,3] *and Nicolae Suciu*[1,4,5]

[1] Alessandrescu-Rusescu National Institute for Mother and Child Health, Fetal Medicine Excellence Research Center, Bucharest, Romania, [2] Independent Researcher, Bucharest, Romania, [3] Department of Cell and Molecular Biology and Histology, Carol Davila University of Medicine and Pharmacy, Bucharest, Romania, [4] Division of Obstetrics, Gynecology and Neonatology, Carol Davila University of Medicine and Pharmacy, Bucharest, Romania, [5] Alessandrescu-Rusescu National Institute for Mother and Child Health, Department of Obstetrics and Gynecology, Polizu Clinical Hospital, Bucharest, Romania

Background: The SARS-CoV2 infection has rapidly spread throughout the world, particularly affecting those with underlying conditions.

Objective: To assess the impact of SARS-CoV-2 on the most prevalent comorbidities, among people who died of COVID-19 in Romania.

Methods: The study comprised 814 deaths caused by COVID-19 between 22nd March and 8th May, 2020 as reported by the Ministry of Health. WHO data regarding deaths of the general population of Romania was used for comparison. The study analyzed the demographics, number and prevalence of comorbidities and calculated the relative risk for each comorbidity.

Results: The study sample consisted of 61.4% males and 38.6% females; the mean age was 68.2 y; 90.9% of deaths occurred in people 50+ years. The mean number of pre-existing conditions was 2.73 (SD = 1.521), with 97.4% of the patients having at least one. The most prevalent comorbidities were hypertension (43.1%), diabetes (33.2%), and coronary heart disease (26.0%). The calculated relative risk of death due to COVID-19 was divided into 3 risk categories: high impact comorbidities (RR > 3) included diabetes RR = 6.426 (95% CI, 4.965–8.318), chronic renal disease RR = 4.338 (95% CI, 3.556–5.292) and hypertension RR=3.261 (95% CI, 2.687–3.958). The medium impact (RR = 2–3) group comprised chronic pulmonary disease RR = 2.615 (95% CI, 2.061–3.319) and chronic liver disease RR = 1.577 (95% CI, 1.183–2.104) and the low impact group (RR<2) –coronary heart disease RR = 0.664 (95% CI, 0.581–0.758), cancer RR = 0.515 (95% CI, 0.416–0.637) and stroke RR = 0.468 (95% CI, 0.370–0.593).

OPEN ACCESS

Edited by:
Zisis Kozlakidis, International Agency For Research on Cancer (IARC), France

Reviewed by:
Lei Huang, University of Massachusetts Medical School, United States
Stefano Perlini, University of Pavia, Italy
Xianding Deng, University of California, San Francisco, United States

***Correspondence:**
Dana Claudia Thompson
dana.lunganu@gmail.com

[†] *These authors have contributed equally to this work*

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: *29 May 2020*
Accepted: *17 August 2020*
Published: *09 September 2020*

Citation:
Barbu MG, Thompson RJ, Thompson DC, Cretoiu D and Suciu N (2020) The Impact of SARS-CoV-2 on the Most Common Comorbidities–A Retrospective Study on 814 COVID-19 Deaths in Romania. Front. Med. 7:567199. doi: 10.3389/fmed.2020.567199

Conclusion: In the studied sample, SARS-CoV-2 had a greater impact on people with diabetes, chronic renal disease and hypertension and a lesser impact on those with coronary heart disease, cancer and stroke. Therefore, future policies in Romania should focus on shielding people in the high-risk group and prioritizing them for vaccination, whilst encouraging those in the low risk group to continue seeking treatment for their underlying diseases.

Keywords: coronavirus, diabetes, hypertension, chronic renal disease, coronary heart disease, cancer, stroke, COPD

INTRODUCTION

Coronaviruses represent a class of RNA viruses that are enveloped, positive-sense and belong to the Coronaviridae family. They can be encountered primarily in human and other mammalian hosts (1). Even though most of the human infections of coronaviruses are mild, they have been responsible for two other notable epidemics in the last two decades that had high mortality rates (10% and 37% for SARS-CoV and MERS-CoV, respectively) and caused a significant number of deaths (2–4).

The latest global pandemic reported its first pneumonia cases of unknown origin in December 2019 in the city of Wuhan, Hubei province, China (4). The virus responsible for it was identified as a novel RNA coronavirus and subsequently named SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2), due to its similarities to the first SARS-CoV (5). From a genetic standpoint, SARS-Cov-2 has been found to have >80% sequence similarity with SARS-CoV and 50% with MERS-CoV (6). The most common symptoms of this condition include fever, cough, myalgia and, to a lesser extent, vomiting or nausea and diarrhea (4, 7). As of May 8, 2020, a number of 4,009,284 cases have been documented globally, with a total of 275,976 reported deaths (8). Out of the infected people, 2,337,180 were still active at that date, with 2,288,477 or 98% in a mild condition and 48,703 or 2% in a serious or critical state (8).

On the same day in Romania, there were 14,811 confirmed cases of SARS-CoV-2 infection and 922 recorded deaths caused by this pathogen (9). However, most of the patients who died had a number of comorbidities associated and, as other previous articles have suggested, some of these diseases could be directly responsible for a proportion of these deaths (10). Furthermore, other studies have also demonstrated an important correlation between certain ailments, like diabetes, and exitus due to the novel coronavirus infection, suggesting that they could represent an important underlying risk factor (11, 12). In this study, we aim to analyze the impact of SARS-CoV-2 infection on the most frequent comorbidities encountered amongst those who have died due to this pathogen in the Romanian population, and how their lifespan was influenced.

MATERIALS AND METHODS

Patients

The first reported case of Covid-19 (Coronavirus Disease 2019) emerged in Romania on 26th February, 2020 in Gorj County, where a 20-year-old male was infected by a 71-year-old Italian man from Cattolica who was diagnosed with coronavirus. Further, on March 22nd, the first three patients died in Romania, all of whom had preexisting conditions.

The Romanian Ministry of Health centralized daily the new information received from hospitals across the country regarding the deceased patients. Furthermore, they provided a daily update, at 1 p.m., about the number of new confirmed cases, the number of people cured and the number of deaths that occurred in the past 24 h, together with a number of details regarding each deceased person. The public communication was done through the Strategic Communication Group and through the official website of the Ministry of Health at http://www.ms.ro/comunicate/. Because the data was made publicly available online, it does not require Institutional Review Board oversight and approval.

The authors took into consideration for this study all the deaths caused by SARS-CoV-2 between 22nd March and 8th May, 2020 that were reported by the Ministry of Health. The available data generally included information about the age, gender, the ward where the patient was admitted, symptoms, the date when the patient was tested, confirmed positive, the date of death and also their underlying comorbidities. All the information was anonymous, with no patient identifiers. Data regarding ethnicity or race was not included in the report, however, according to the last available census, 88.9% of the total population was represented by Romanians, 6.5% were Hungarians, 3.3% were Romani, while the rest comprised other minorities, such as Ukrainians, Germans, Turks and Russians. Information about race was not covered by this census (13). In order to be included in the study, the minimum amount of details considered essential by the authors consisted of age, gender and comorbidities (including whether they had them or not). Therefore, after manually selecting each case, out of the total number of 922 reported deaths on May 8th, only 814 met the abovementioned criteria.

From the 108 people excluded, 54.6% were from Suceava, Arad or Ialomita, where there were known outbreaks and where, because of the high number of deceased, the doctors did not always report all the details about them. Furthermore, there were a number of people that did not have a family doctor or whose records were in overseas territories, for whom it was stated that the comorbidities were not known. Finally, there were also people that have died in care homes and whose medical records were not known.

According to law number 436 from 13th March 2020, which was later updated on 6th April 2020, both being published in the Official Monitor, the protocol regarding the diagnostics of death from SARS-CoV-2 states that there are a number of situations when a doctor can write Covid-19 as the main cause on the death certificate (14, 15).

- All the patients that have died while they were hospitalized and were diagnosed with the coronavirus infection during their stay will have Covid-19 considered as their main cause of death. An autopsy will not be performed because of the infection risk for the medical personnel, unless it is done for a scientific scope or it is considered a forensic case.
- All the people that have died while in self-isolation or in quarantine upon their return from countries considered at risk will be classified as deaths due to Covid-19 and confirmed as being positive for coronavirus infection.
- All the people that have died while in hospital and were suspected to have been infected with coronavirus, but did not get a confirmation test, will be considered deaths caused by SARS-Cov-2.

The Romanian law is in accordance with the WHO International Guidelines for Certification and Classification (Coding) of Covid-19 as Cause of Death, which also states that "individual countries should not correct what is assumed to be an error, since changes at the national level will lead to data that are less comparable to data from other countries, and thus less useful for analysis." Therefore, the authors cannot state that all of the patients who were officially reported as Covid-19 deaths by the Ministry of Health had a positive SARS-CoV-2 test, as they were not necessarily required one in order for this diagnosis to be considered on their death certificates (16).

Terms and Measures

In this study, the authors aimed to look into the impact of the new coronavirus over certain comorbidities in a cohort of people that have died from Covid-19, how their lifespan was influenced and whether it suffered a reduction or was simply not affected. In order to achieve that desiderate, the group of Covid-19 deaths reported by the Ministry of Health was compared to the percentages of deaths caused by the studied comorbidities in the general population of Romania. However, a significant number of people that have died from the coronavirus group had multiple comorbidities, while the analysis on the general population was done by a singular cause of death.

To be able to make an appropriate comparison, a 3 stage selection criteria was put in place. First, it was taken into consideration the ward through which the patient arrived at the hospital or the train of events that led to the intake of the patient, indicating that the respective affliction was in an acute state and could have posed a potentially greater risk of exitus at that time. If the patient came through the emergency room, it was considered that for each subject in the coronavirus group, the comorbidity that would have had the highest impact on their overall life expectancy would be the one with the lowest 5 year survival rate. Therefore, a literature review was performed and a ranking system was put in place for these cases. Lastly, if the comorbidity was known for causing deaths indirectly and a 5 year life expectancy could not be found, the selection was made according to the number of reported deaths caused by it within the Romanian population. The summary of disease severity ranking considered by the authors for this study, in the absence of Covid-19, is summarized in **Table 1**. Further on, the 5 year survival rate is detailed for each disease.

- *Coronary Heart Diseases* (CHD) are considered the number one cause of mortality worldwide, having a particularly high impact on the Romanian population. They are responsible for over 9 million deaths per year across the globe, according to the World Health Organization (WHO) (44). Even though their mortality has progressively decreased over the last years in Western countries, thanks to the advancements in primary prevention and the improvement of diagnosis and treatment, they still represent a significant burden for developing countries (45). The 5 year survival rate after suffering an acute myocardial infarction was found to be around 56%, with 27.1% dying within a 30 day interval and 23.7% beyond that (41). Diabetes, stroke, heart failure and obesity (BMI>30) showing adjusted hazard ratios (AHR) of 1.83 (95% CI, 1.43–2.34), 1.73 (95% CI, 1.35–2.22), 1.69 (95% CI, 1.28–2.22), and 1.39 (95% CI, 1.01–1.90), respectively, were also shown to increase the mortality of CHD independent of other risk factors (41).
- *Cancer* represents the second cause of morbidity and mortality in the world. Despite the fact that Europe only represents 9% of the world population, the WHO has reported that 20.3% of the total number of deaths through cancer are encountered here (46). In Romania, cancer of all causes is responsible for ∼20% of deaths, the most frequent types being lung cancer, colon cancer, breast cancer and stomach cancer (47). Some of the 5 year survival rates for the types of cancer encountered in our cohort are: for non-small cell lung cancer−61% for localized cancer, 35% for regional and 6% for distant, with an all stages combined average of 24%; for small-cell lung cancer−27% for localized cancer, 16% for regional and 3% for distant, with an all stages combined average of 6%; for colon cancer−90% for localized cancer, 71% for regional and 14% for distant, with an all stages combined average of 63%; for breast cancer−100% for Stages 0 and I, 93% for Stage II, 72% Stage III, and 22% Stage IV; for acute lymphoblastic leukemia−70% for people aged 15–24, 40% for people aged 25–64 and 15% for people over 65 years old. Because of the high number of different types of neoplasms, the rest will be summarized in **Table 1**.
- *Stroke* is also one of the leading causes of death around the world. In Romania, it is responsible for ∼17% of all deaths (47). The 5 year survival rate for stroke was reported to be 58.3% across all types, with 59.2% of ischemic strokes surviving after 5 years, 55.4% of intracerebral hemorrhages and 55.2% of subarachnoid hemorrhages, respectively (42).

TABLE 1 | Five year survival rates by disease type and subtype.

Category	Disease subtype	5 Year survival rates	References
Cancer	Mediastinum cancer–distant metastasis	0%	(17)
	Pancreas cancer stage 4	3%	(18)
	Cancer—SLC	6%	(19)
	Cancer–Leukemia (65+)	15%	(20)
	Rectal cancer—distant	15%	(21)
	Ovarian cancer–stage IV	17%	(22)
	Liver cancer	18%	(23)
	Stage 4 cervical cancer	20%	(24)
	Cancer–Breast Stage 4	22%	(25)
	Cancer—NSLC	24%	(19)
	Melanoma—distant	25%	(26)
	Gastric cancer—spread	31%	(27)
	Waldenstrom Disease—severe	36%	(28)
	Neuroendocrine tumors	39%	(29)
	Cancer–Leukemia (25–64)	40%	(20)
	Laryngeal cancer—regional	45%	(30)
	Mediastinum cancer–pulmonary metastasis	45%	(17)
	Myeloid metaplasia	50%	(31)
	Multiple myeloma—overall	52%	(32)
	Meningioma malignant	55%	(33)
	Cancer—colon	63%	(21)
	Melanoma—regional	65%	(26)
	Oropharyngeal cancer—overall	65%	(34)
	Rectal cancer—overall	67%	(21)
	Duodenal cancer—overall	68%	(35)
	Gastric cancer—localized	69%	(27)
	Cancer–Leukemia (15–24)	70%	(20)
	Chronic Leukemia over 75 years old	70%	(36)
	Meningioma benign	70%	(33)
	Rectal cancer—regional	71%	(21)
	Cancer–breast stage 3	72%	(25)
	Mediastinum cancer—localized	72%	(17)
	Meningioma atypical	75%	(33)
	Uterine cancer	75%	(37)
	Chronic Leukemia (under 75 years old)	83%	(36)
	Rectal cancer—localized	89%	(21)
	Melanoma—overall	92%	(26)
	Cancer–breast stage 2	93%	(25)
	Prostate cancer—overall	98%	(38)
	Melanoma—localized	99%	(26)
	Cancer–Breast Stage 0 & 1	100%	(25)
Chronic liver disease	Chronic Liver Disease (Hospitalized)	33%	(39)
	Chronic liver disease (ambulatory)	66%	(39)
Chronic pulmonary disease	COPD	26%	(40)
Coronary heart disease	Coronary heart disease	56%	(41)
Stroke	Stroke	58%	(42)
Chronic renal disease	Renal disease–end stage (dialysis)	35%	(43)
	Renal disease–end stage (after transplant)	80%	(43)

- *End Stage Renal Disease* is estimated to affect around 2 million people worldwide, with an increase of 5–7% per year. There are only two treatment options for these patients at the moment, which are transplantation or dialysis. Transplantation can be made with a kidney from a living or deceased donor and it leads to a 5 year survival rate of over 80%. However, compatible donors are difficult to find and most patients will require dialysis. This can be done in two ways—hemodialysis and peritoneal dialysis, with the vast majority (over 90%) belonging to the first group. The 5 year survival rate for patients on dialysis is 35% overall (43).
- *Chronic Pulmonary Diseases* (CPD) are represented by asthma and chronic obstructive pulmonary disease (COPD). COPD is currently the third leading cause of death around the world, with 384 million people suffering from this disease and 3 million people dying each year. At the same time, 334 million people are known to be diagnosed with asthma, therefore making it the most prevalent chronic childhood disease (48). Previous studies have shown that the 5 year survival rate for COPD after the first episode of acute exacerbation was 26% (40). In the case of asthma, the authors could not find a 5 year survival figure, therefore the severity was determined according to the number of deaths caused by asthma in Romania in one year.
- *Chronic Liver Disease* (CLD) is another global health challenge and it is defined as a hepatic suffering for more than 6 months. In this case, studies found that the 5 year survival rate was around 47%, for all causes of cirrhosis, while the average survival probabilities at 5 years were 0.66 (95% CI, 0.63–0.68) for ambulatory treated patients and 0.31 (95% CI, 0.29–0.33) following hospitalization (39).
- *Diabetes* and *Hypertension* were considered in most cases to be predictors of mortality for the abovementioned diseases. In the cases where they could not be associated with another illness, the difference between the two was made based on the number of deaths caused by each one in the Romanian population. The results lead to the conclusion that hypertension should be placed ahead of diabetes, with a number of 8,900 reported deaths caused by it in one year, against 2,700 caused by diabetes (47).

Procedures

After collecting the data and categorizing it according to the abovementioned criteria, the authors proceeded to analyze it. In order to facilitate the understanding, a more visual approach was taken by creating several charts and tables. As a first step, the group of people deceased from SARS-CoV-2 was separately described.

The gender distribution was evaluated and then placed side by side with the gender distribution of deaths in the general population of Romania. Then, the age distribution was considered and also the distribution of the number of comorbidities. For these two, histograms shaped as bell curves were created, thus displaying the mean, median and mode values, together with the standard deviation. The last step was performed using the latest version of SPSS Statistics software package, developed by IBM. Further on, the cumulative number

of comorbidities was calculated and converted into percentages, for both ascending and descending values and after that the prevalence of each comorbidity in the studied cohort was assessed and turned into a bar chart.

After having completed the first step and once all the data from the Covid-19 group were summarized, the study moved on to the second stage, which involved the statistical analysis of the chosen comorbidities in comparison to the deaths in the general population. The sample consisted of 814 cases which met all the necessary inclusion criteria. Out of these, 654 people had at least one of the chosen comorbidities, 22 people had no comorbidities and 137 people had other comorbidities that were not analyzed due to insufficient cases for each comorbidity to have statistical significance.

Statistical Analysis

The second step consisted of two different statistical analyses. The first question that was answered was whether a correlation between the age and the number of comorbidities existed in the Covid-19 group. In order to achieve this, a scatter plot was created and the coefficient of determination (R square) was assessed. In this case, the R square was applied to a linear regression model that had only one independent variable (the age), therefore the following formula was used:

$$R^2 = \left\{ \left(\frac{1}{N} \right) \times \sum \frac{\left[(x_i - \overline{x}) \times \left(y_i - \overline{y} \right) \right]}{\left(\sigma x \times \sigma y \right)} \right\}^2$$

Where N was the number of observations, Σ was the symbol of summation, x_i was the value of x for observation i, $\overline{x}$ was the mean of all the x values, y_i was the value of y for observation i, $\overline{y}$ was the mean of all the y values and σx and σy are the standard deviations of x and y, respectively (49).

The next question was whether the novel coronavirus impacted certain comorbidities and by how much was the risk of death increased for the people infected, compared to the number of deaths caused by these diseases under normal circumstances. To answer this, the authors first applied the Pearson's chi-squared test to assess if in the sets of categorical variables used, any observed differences between them was due to chance. The mathematical formula for this was:

$$x_c^2 = \sum \frac{(O_i - E_i)^2}{E_i}$$

Where c represented the degrees of freedom, O were the observed values and E were the expected values. A value of $p \leq 0.05$ was indicative of statistical significance.

Further on, in order to quantify the effect of SARS-CoV-2 over the risk of death by having certain comorbidities associated, the authors computed the relative risk (RR), the odds ratio (OR), the attributable risk (AR), and the attributable risk percent (AR%). For all of their values, a 95% confidence interval (CI) was considered, and the lower and upper limits of the interval were presented. The formulae were calculated given the following example of a contingency table (**Table 2**):

TABLE 2 | Contingency table to showcase the formulae for RR, OR, AR, and AR%.

Factors	Comorbidity		Total
	Yes	No	
SARS-CoV-2 exposed group	A	B	a+b
SARS-CoV-2 non-exposed group	C	D	c+d

$$Relative\ Risk\ (RR) = \frac{\left(\frac{a}{a+b} \right)}{\left(\frac{c}{c+d} \right)}$$

$$Odds\ Ratio\ (OR) = \frac{\frac{a}{b}}{\frac{c}{d}}$$

$$Attributable\ Risk\ (AR) = \left(\frac{a}{a+b} \right) - \left(\frac{c}{c+d} \right)$$

$$Attributable\ Risk\ Percent\ (AR\%) = \frac{\left(\frac{a}{a+b} \right) - \left(\frac{c}{c+d} \right)}{\left(\frac{a}{a+b} \right)} \times 100$$

The statistical analysis was conducted using IBM SPSS Statistics for Windows, Version 26.0 (IBM Corp., Armonk, NY, USA).

RESULTS

The total number of reported deaths due to SARS-CoV-2 infection until the 8th of May was 922, out of which 13 were excluded because the data did not contain age and/or gender information. Further, 95 reported cases lacked information about preexisting comorbidities, leading to a sample size of 814 patients, which was analyzed for age, gender and the number of comorbidities distribution. Out of this sample, 21 patients had no preexisting conditions and 139 had various others that were not included in the study because the preliminary data did not result in significantly statistical information, meaning that the sample analyzed for the prevalence of comorbidities consisted of 654 cases (**Table 3**). Most of the excluded cases for lack of information pertained to highly infected areas where probably not enough time was available for thorough anamnesis and/or data uploading.

Sample Data

After the exclusion criteria detailed above were applied, the COVID-19 study group consisted of 814 patients that were reported to have died because of SARS-CoV-2 infection between 22nd March and 8th May 2020, out of which 500 were males (61.4%) and 314 were females (38.6%) (**Figure 1**).

Further, we compared the obtained results with the most recent statistical data of the general Romanian population deaths provided by the WHO, which showed a slightly higher percentage of female deaths (51.5 vs. 48.5%) in the year 2016. Thus,

TABLE 3 | Sample sizes and exclusion criteria.

Sample category	Frequency	Percent	Mean age	Mean no. of comorbidities	% Male
Total deaths in Romania	922	100%	–	–	–
Total with age & gender info	**909**	**98.6%**	**68.1**	–	**61.4%**
(Those without age & gender)	13	1.4%	66.5	–	–
Total with comorbidity info listed*	**814**	**88.3%**	**68.3**	**2.73**	**61.4%**
(Those without comorbidity info listed)	95	10.3%	66.7	–	61.1%
Total with studied comorbidities**	**654**	**70.9%**	**68.7**	**2.87**	**61.6%**
(Those with other comorbidities)	139	15.1%	67.7	2.52	58.3%
(Those recorded with no comorbidities)	21	2.3%	59.0	–	76.2%

** This figure was used for all the analyses except prevalence.*
*** Total used for the study of disease prevalence.*

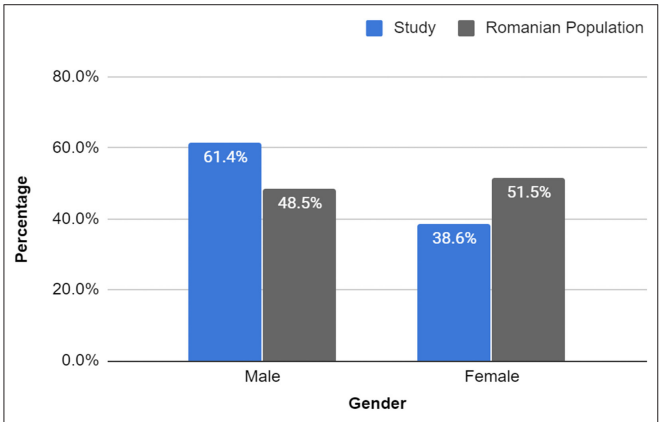


FIGURE 1 | Gender percentage of study sample vs. Romanian population. The percentages of male and female deaths were clearly different from those of the general population. Study: Male (500): 61.4%, Female (314): 38.6% vs. Romanian Population: 48.5 and 51.5%, respectively. Male RR = 1.268 (95% CI, 1.200–1.339), $p < 0.0001$; Female RR = 0.748 (95% CI, 0.686–0.816), $p < 0.0001$. Source of Romanian Population Deaths: WHO Member States 2016 (47).

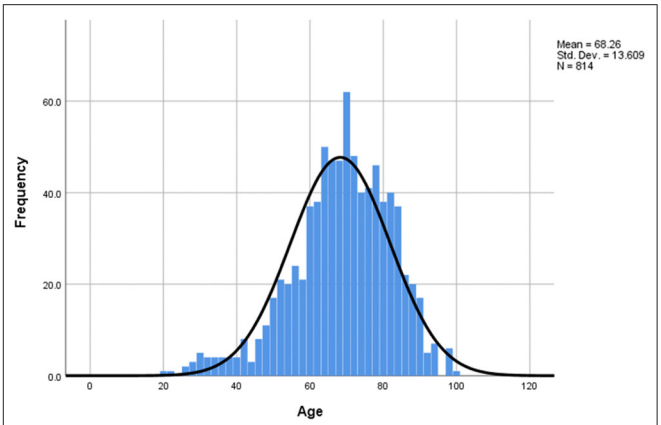


FIGURE 2 | Age distribution of study sample. The mean age of death = 68.26 y, median = 69 y, mode = 70 y, min = 20 y, max = 98 y, SD = 13.609. Distribution by age intervals: 0–19: 0%, 20–29: 0.9%, 30–39: 2.9%, 40–49: 5.3%, 50–59: 13.3%, 60–69: 28.0%, 70–79: 28.3%, 80–89: 18.1%, 90–99: 3.3%. 77.6% of deaths were over 60 (22.4% under) and 90.9% of deaths were 50+ (9.1% under 50).

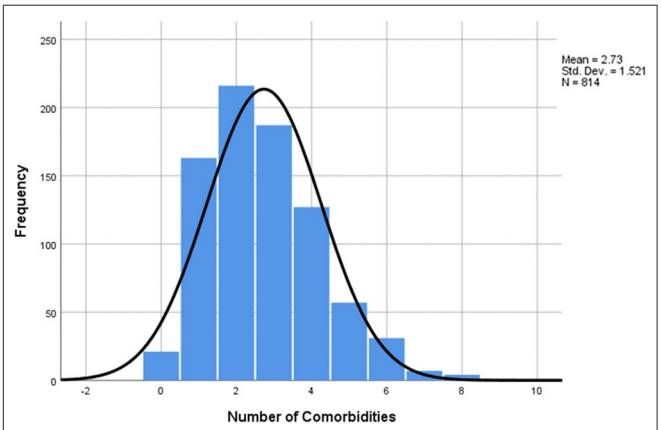


FIGURE 3 | Number of comorbidities distribution of study sample. The mean number of comorbidities was 2.73, median = 3, mode = 2, min =0 (21 patients), max =9 (1 patient), SD = 1.51.

the relative risk of each gender dying due to COVID-19 was calculated and it was discovered that males had an RR of 1.27 (95% CI, 1.200–1.339), $p < 0.0001$, meaning that they had a 27% increase in the risk of death in the event of SARS-CoV-2 infection, while females had a RR of 0.75 (95% CI, 0.686–0.816), $p < 0.0001$, translating into a 25% decrease in the risk of COVID-19 exitus.

The age distribution of the COVID-19 study group was analyzed and it was observed that the mean age of death due to SARS-CoV-2 infection was 68.26 y (SD 13.609) (**Figure 2**), being slightly higher for females (70.2 y) compared to males (67.1 y). Out of the 814 patients studied, 740 deaths (90.9%) occurred to those over 50 y. The percentages for each age group can be found in the description of **Figure 2** below.

In the next step, the distribution of the number of comorbidities was taken into consideration, and it was found that the mean number of underlying diseases was 2.73 (SD=1.521) (**Figure 3**), with one patient having nine comorbidities reported and 21 patients having none. Furthermore, the cumulative frequency by number of comorbidities was calculated and the data obtained showed that 793 patients (97.4%) had at least one underlying ailment, while 415 patients (50.9%) had three or more (**Table 4**). Additional data on the cumulative frequency of comorbidities can be found in **Table 4**.

TABLE 4 | Cumulative frequency by number of comorbidities of study sample.

No. of comorbidities	Frequency	Percent	Cumulative percent	Inverted cumulative
0	21	2.6%	2.6%	100.0%
1	163	20.0%	22.6%	97.4%
2	215	26.4%	49.0%	77.4%
3	187	23.0%	72.0%	50.9%
4	127	15.6%	87.6%	27.9%
5	57	7.0%	94.6%	12.3%
6	31	3.8%	98.4%	5.3%
7	8	0.9%	99.3%	1.5%
8	4	0.5%	99.8%	0.6%
9	1	0.1%	99.9%	0.1%
Total	**814**	**100.0%**	**100.0%**	**100.0%**

As mentioned in the beginning of the Results section, out of the total of 814 cases studied, 21 more patients were excluded for the comorbidity prevalence analysis because they had no preexisting condition and 139 had other comorbidities than those studied, leading to a sample size, for this particular analysis only, of 654 cases. Hypertension (43.1%), diabetes (33.2%), and coronary heart disease (26.0%) were the three most prevalent preexisting conditions among the patients that died due to SARS-CoV-2 infection, while cancer (10.9%), stroke (9.8%) and chronic liver disease (9.7%) were the least prevalent (**Figure 4**).

Further, after applying the criteria for the most severe comorbidity, the prevalence of comorbidities in the study group, which included those without preexisting conditions and patients with other diseases (814 cases), was compared to the mortality data provided by the WHO for the general population of Romania. As can be seen in **Table 5**, some of the comorbidities, such as hypertension (11.3 vs. 3.5%), chronic renal disease (10.8 vs. 2.5%), chronic pulmonary disease (7.7 vs. 3.0%) and diabetes (6.8 vs. 1.1%) had higher frequencies in the COVID-19 group than in the general population. In contrast, coronary heart disease (21.0 vs. 31.7%), cancer (9.5 vs. 18.4%) and stroke (7.9 vs. 16.8%) were less frequent in the study group than in the general population (**Table 5**). Small differences were observed in the percentages of those with no comorbidities (2.6 vs. 3.8%) and those that had other conditions (17.1 vs. 16.0%). For comparison, the authors further included a table comprising 10 other relevant countries by region, showcasing the difference in prevalence amongst the people that have died of these particular diseases (**Table 6**). The countries were selected taking into consideration the quality of the reported data, as per the WHO assessment (47). The table shows that Romania fits into the central and eastern European model, having similar figures to Russia, with higher than average rates for coronary heart diseases and stroke and a lower incidence of diabetes and chronic pulmonary disease. To ascertain the relative risk of each comorbidity by country, a further analysis should be done within that country's Covid-19 data set.

Obesity was also a potential risk factor but was not included as part of the studied comorbidities, as it was classified together with other metabolic disorders as a cause of death in the WHO reference data. 137 patients (16.8% of 814) had obesity listed on their death certificates in total and 37 patients had obesity in the other comorbidities group (12.2% of 139). The average age of death for those with obesity was 58.8 y, while those with normal weight had an average age of 70.2 y. Obesity was present in 40.5% of cases for those under 50 y and in 14.5% for those over 50 y.

Statistical Analysis

In order to assess the relationship between the number of comorbidities and age, a linear regression model was applied, illustrated in **Figure 5**. The findings showed that the number of comorbidities did increase with age, however the R squared value indicated to us that age was not a major influencing factor for the number of underlying diseases. In this case, the R squared value was 0.035, meaning that in only 3.5% of cases the correlation between age and comorbidities was positive. The overall mean number of comorbidities was 2.73 (SD=1.521), with a slightly lower average observed in younger ages (39, 40, 42, 43, 46–51) than in older patients (80–89)−2.7 vs. 3.0 (**Table 7**).

Further, the Pearson's chi-squared test was applied in order to evaluate if the association between the two variables was a random event. A value under 0.05 was considered statistically significant, which in our case was valid for all the comorbidities studied. After that, the relative risk of exitus for each disease in the SARS-CoV-2 group was computed against that of the general population of Romania, using the most recent statistical data provided by the WHO, from the year 2016. The results showed an increased relative risk for some of the ailments, such as diabetes −6.426 (95% CI, 4.965–8.318), chronic renal disease −4.338 (95% CI, 3.556–5.292), hypertension −3.261 (95% CI, 2.687–3.958), chronic pulmonary disease −2.615 (95% CI, 2.061–3.319) and chronic liver disease −1.577 (95% CI, 1.183–2.104), while for others there was a lower risk, as in the case of coronary heart disease −0.664 (95% CI, 0.581–0.758), cancer −0.515 (95% CI, 0.416–0.637), and stroke −0.468 (95% CI, 0.370–0.593) (**Table 8**). For all the relative risk values above mentioned, the p-value was <0.05. The calculated odds ratios also supported the previously described findings (**Table 8**).

For the next step, the COVID-19 group was divided into 3 categories, according to the relative risk, as following: for the diseases that had a relative risk over 3 it was considered that the coronavirus had a high impact on mortality, the ones with a relative risk between 1 and 3 were considered medium impact, while those below 1 were considered low impact. In order to determine the negative influence of SARS-CoV-2 over these comorbidities, the attributable risk was calculated for each of them, together with the percentage that could have been prevented if the infection had not occurred (attributable risk percent). Lastly, the mean age for each group was also determined and added into **Table 9**. The average attributable risk (AR) for the COVID-19 high impact group was 0.219 (95% CI, 0.201–0.236), with an AR% of 75.7% (95% CI, 69.6–81.8%) and a mean age of 66.0. For the COVID-19 medium impact group, the AR was 0.068

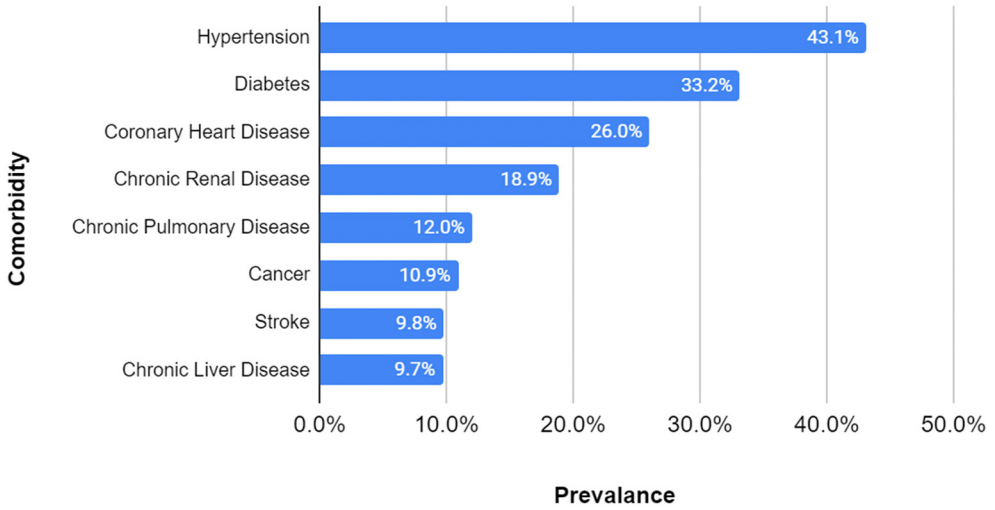


FIGURE 4 | Comorbidity prevalence in study group. Sample size = 654 (108 excluded for lack of information, 21 had no comorbidities, and 139 had other comorbidities). The most prevalent three comorbidities were hypertension, diabetes and coronary heart disease, while cancer, stroke, and chronic liver disease were the least prevalent.

TABLE 5 | Death frequency by comorbidity for study group and Romanian population.

Most severe comorbidity	Study sample		Romanian population	
	Frequency	%	Deaths ('000)	%
Coronary heart disease	171	21.0%	81.3	31.7%
Hypertension	92	11.3%	8.9	3.5%
Chronic renal disease	88	10.8%	6.4	2.5%
Cancer	77	9.5%	47.2	18.4%
Stroke	64	7.9%	43.1	16.8%
Chronic pulmonary disease	63	7.7%	7.6	3.0%
Diabetes	55	6.8%	2.7	1.1%
Chronic liver disease	44	5.4%	8.8	3.4%
No comorbidities*	21	2.6%	9.8	3.8%
Other comorbidities	139	17.1%	41.0	16.0%
Total**	814	100.0%	256.8	100.0%

This included injuries and substance abuse.
***500 neonatal and maternal deaths were excluded from the data set for the Romanian population, as they were not relevant for this study.*
Source of Romanian Population Deaths: WHO Member States 2016 (47).

(95% CI, 0.051–0.084), with an AR% of 51.4% (95% CI, 38.6–64.2%) and a mean age of 64.8, whereas for the low impact group, the AR was −0.285 (95% CI, −0.317– −0.253), AR% was −74.3% (95% CI, −82.8%– −65.9%) and a mean age of 72.0. It can be observed that the mean age of death was lower in the first two risk categories, compared to the low impact one. All the obtained results can be found in **Table 9**.

In addition, for the patients with no comorbidities, the cause of death was attributed entirely to the SARS-CoV-2 infection. The group with other comorbidities than the ones studied were not taken into consideration due to the fact that the *p*-value was above 0.05.

DISCUSSION

Most of the results obtained in this study were in accordance with previous research regarding the SARS-CoV-2 infection. Pertaining to the gender, the findings showed that more deaths occurred amongst men (61.4 vs. 38.6%), in comparison to the deaths in the general population, which seemed to be more balanced and more increased for women (48.5 vs. 51.5%). A prior retrospective study, conducted on 168 patients, also revealed that men were more predisposed to severe outcomes, such as death (12.8 vs. 7.3%), in comparison to women and were more prone to develop critical illness. This might be explained by the fact that the male gender seemed to be more at risk when associating comorbidities, than women (OR = 3.824, 95% CI = 1.279–11.435 vs. OR = 2.992, 95% CI = 0.937–9.558) (50). Similar outcomes have been observed in the case of MERS-CoV and SARS-CoV infections (51, 52). The mechanism why men are more prevalent in the deceased group is not clear, but one possible explanation could be attributed to steroid hormones and X chromosome genes, both of which were previously shown to regulate the immune response in viral infections (53, 54). Another cause that should be considered is the fact that men are less likely to seek early medical consultations for the common diseases, which could have led to more severe states of their underlying conditions (55).

Regarding age, 77.6% of the studied group were over 60 years old, which is in alignment with reported data from China, where 80% of deaths occurred in the 60+ age group (56). Furthermore, the mean age of death in the study group was 68.26 y (SD = 13.609), with a mean age for females of 70.2 y and for males of 67.1 y. In comparison, the average life expectancy provided by the WHO for Romania was 75.2 y, 71.6 y for males, and 79.0 for females (47). Perhaps the lower means could be explained by the coronavirus infection in Romania, however it should

TABLE 6 | Death frequency by comorbidity for Romania vs. ten other countries.

Region	World avg.	Romania	Australia	Brazil	China	Israel	Italy	Japan	Russia	South Africa	United Kingdom	USA
	%	%	%	%	%	%	%	%	%	%	%	%
Coronary heart disease	17.4	31.7	14.9	13.2	18.7	11.7	17.6	11.7	32.1	8.2	12.8	17.9
Cancer*	15.0	18.4	26.2	16.3	21.7	24.8	24.4	26.9	17.4	9.3	25.6	20.5
Stroke	10.7	16.8	6.8	8.6	19.6	5.6	9.7	9.6	16.5	6.0	6.4	5.3
Chronic pulmonary disease**	6.4	3.0	5.9	5.0	8.9	4.5	5.0	5.2	1.7	4.2	6.5	7.1
Diabetes	2.9	1.1	3.0	5.0	1.6	6.1	3.5	1.1	0.5	7.3	1.1	3.0
Chronic liver disease	2.3	3.4	1.0	1.9	1.5	0.9	1.4	1.3	2.2	1.1	1.3	1.8
Chronic renal disease	2.2	2.5	1.6	2.0	1.9	4.1	2.3	2.7	0.5	2.2	0.8	2.4
Hypertension	1.7	3.5	0.8	1.6	2.7	1.4	2.8	0.3	1.1	2.4	0.6	1.7
Other comorbidities	32.2	16.0	33.0	33.8	16.1	36.6	29.6	36.3	19.1	49.5	41.0	32.4
No comorbidities***	9.3	3.8	6.7	12.5	7.3	4.3	3.9	4.9	8.8	9.6	3.9	7.9

*Includes cancers present among patients in this study, as listed in **Table 1**. Other cancers are included in Other Comorbidities.*
***Includes COPD and Asthma.*
****Includes deaths from injury and substance abuse.*
Neonatal and maternal deaths have been excluded for the total deaths, as they are not relevant for this study.
Countries were selected in each world region, to give a diverse range, based on a balance of the quality of data as determined by the WHO, population size and covid-19 deaths.
The world average includes countries with good and poor quality data, as judged by the WHO, so should be considered with caution.
Source: WHO Member States 2016 (47).

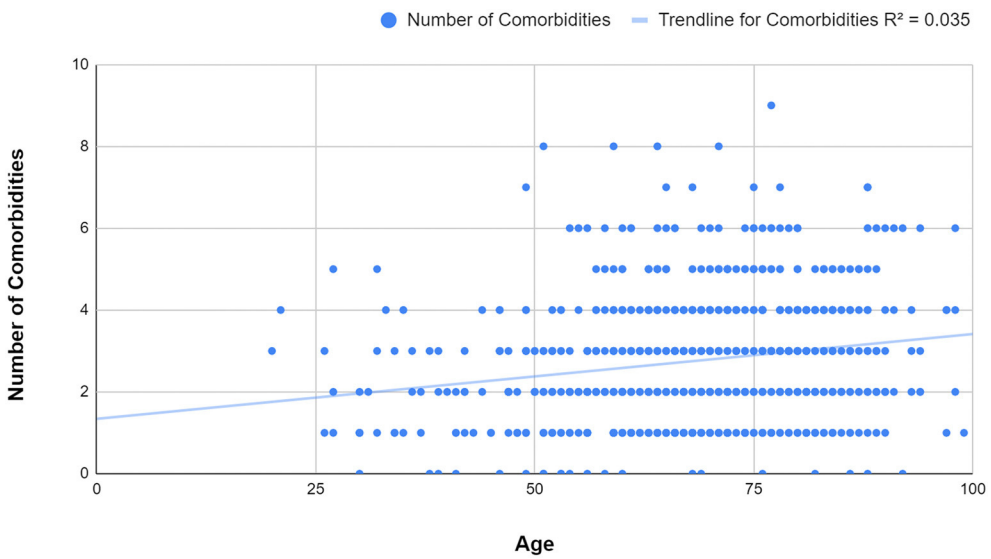


FIGURE 5 | Figure 5 Linear regression scatter plot of age and number of comorbidities. It can be observed that the number of comorbidities slightly increases with age (R squared = 0.035).

be noted that the WHO life expectancy data is from 2016, therefore it refers to the population born in that year. For a more accurate comparison, previous information on life expectancy should be used, altogether with proper adjustments for medical advancements regarding diagnostics and treatment. For more thorough insights, a separate multinational study should be conducted on this matter.

The number of comorbidities analysis showed a mean number of 2.73 (SD=1.521), with a cumulative frequency of underlying diseases of 97.4% for at least one preexisting condition and 50.9% for 3 or more. A previous study conducted in the Wuhan province of China on 1,590 confirmed COVID-19 patients discovered that 25.1% of them had at least one comorbidity, while 8.2% had at least two. They also pointed out that the patients with two or more conditions were more likely to have a severe outcome. After comparing these two groups with the patients without comorbidities, they reached an HR of 1.79 (95% CI, 1.16–2.77) for the people with at least one underlying condition and 2.59 (95% CI, 1.61–4.17) for those with two or more (12). These figures suggest that not only is the presence of comorbidities an influencing factor for the outcome, but also their number plays a proportional part in determining the severity of evolution.

TABLE 7 | Mean number of comorbidities by age.

Age	Frequency	Mean No. comorbidities	Standard deviation
0–19	0.0%	0.0	0.0
20–29	0.9%	2.71	1.50
30–39	2.9%	1.92	1.35
40–49	5.3%	2.12	1.37
50–59	13.3%	2.38	1.55
60–69	28.0%	2.69	1.46
70–79	28.3%	2.93	1.54
80–89	18.1%	2.99	1.46
90–99	3.3%	3.11	1.76
Total	100%	**2.73**	1.52

Similar to other studies (12), the linear regression analysis showed a low strength of association between the number of comorbidities and age (R squared = 0.035), with a relatively even distribution of comorbidities throughout the age groups. This could suggest that even though some deaths occurred in younger people, they were very likely to have had at least one underlying condition. The most prevalent disease amongst the studied group was hypertension (43.1%), followed by diabetes (33.2%) and coronary heart disease (26.0%). These results were very close to the ones found by another already published article, that showed a prevalence of 48% for hypertension, 31% for diabetes and 24% for coronary heart disease (57). Although this could be an explicable encounter for coronary heart disease, which, as mentioned in the literature review, represents the number one cause of mortality worldwide, the other two have a considerably higher prevalence in the coronavirus affected population. Further on, statistical tests were applied and the results are next to be discussed.

The statistical analysis applied to the most common comorbidities encountered in the study group revealed an increased relative risk for some of the ailments, while others were at a lower risk compared to the general population. To ease understanding, the authors decided to divide the study sample into three categories, based on the relative risk value. The diseases considered at high risk were diabetes RR=6.426 (95% CI, 4.965–8.318), chronic renal disease RR=4.338 (95% CI, 3.556–5.292), and hypertension RR=3.261 (95% CI, 2.687–3.958), the ones that displayed a medium risk were chronic pulmonary disease RR=2.615 (95% CI, 2.061–3.319) and chronic liver disease RR=1.577 (95% CI, 1.183–2.104) and the low risk group included coronary heart disease RR=0.664 (95% CI, 0.581–0.758), cancer RR=0.515 (95% CI, 0.416–0.637), and stroke RR=0.468 (95% CI, 0.370–0.593).

Similar to our findings, other studies have looked into the association between the SARS-CoV-2 infection and the high risk comorbidities found in this article. Several papers conducted meta-analyses on the relationship between diabetes and Covid-19 related mortality, showing that the presence of diabetes mellitus increased these patients' risk of in-hospital deaths (OR=1.90 and RR=2.12, respectively) and also enhanced the severity and disease progression of Covid-19 (OR=2.75 and RR=3.31) (58, 59). Chronic renal disease was also found to negatively influence the outcome and progression of SARS-CoV-2 infection, with prior meta-analyses showing a hazard ratio (HR) of in-hospital death as follows: HR=2.10 for patients with increased baseline serum creatinine; HR=3.97 for increased blood urea nitrogen; HR=1.90 for patients suffering from stage 1 acute kidney injury, HR=3.51 for stage 2 acute kidney injury and HR=4.38 for stage 3 acute kidney injury, respectively (60–63). Hypertension was one of the most studied comorbidities affected by the novel coronavirus, having multiple meta-analyses that focused on identifying the effect it had on the outcome. Results indicated OR values of 3.36 for elevated mortality risk in hypertensive patients in comparison to normotensive and an OR of 2.49 for the occurrence of a severe form of Covid-19 (64, 65).

The mean calculated ages were 66.0 (SD=13.32) for the high risk group, 64.8 (SD=12.92) for the medium risk and 72.0 (SD=12.92) for the low risk sample. Instead of following an ascending trend from the high to the low risk group, the mean age was found to be lowest in the medium risk group. One possible reason that the authors found, at least in the case of chronic liver disease, was that it has a naturally lower average age of death, even in the absence of SARS-CoV-2 infection. A nationwide analysis performed in Brazil between the years 2000 and 2012, which included 265,180 deaths due to cirrhosis, found that people suffering from this condition had a median age at the time of death of 56 years (95% CI, 47–67) (66).

Prior research looked into the reasons why diabetes, hypertension and chronic renal disease consistently displayed a higher mortality rate amongst the people infected with SARS-CoV-2. One of the common traits that these three diseases and SARS-CoV-2 share is the fact that angiotensin-converting enzyme 2 (ACE2) is involved in their pathogenesis. ACE2 can be found in lungs, kidney, blood vessels and intestine. It was observed that SARS-CoV and SARS-CoV-2 used ACE2 in order to attach to their host cells. It is also well-known that higher levels of ACE2 can be detected in diabetes, hypertension and chronic renal disease patients, who can benefit from ACE2 inhibitor therapy (67, 68). Further research on this matter needs to be undertaken in order to reveal the exact mechanisms that cause these afflictions to be more at risk.

Finally, the authors calculated the attributable risk percentage (AR %) for the infected group against the general population for each comorbidity. The scope was to assess the percentage of deaths that could have been avoided if the SARS-CoV-2 infection had not occurred, for the diseases studied. The results were AR% = 75.7% (95% CI, 69.6–81.8%) for the high risk group, AR% = 51.4% (95% CI, 38.6–64.2%) for the medium risk one and AR% = −74.3% (95% CI, −82.8–−65.9%) for the low risk group. The respective values for each affliction can be found in **Table 9**. Assuming that the risk that can be attributed to the SARS-CoV-2 infection in the case of people with no comorbidities was 100%, the authors then multiplied the AR% value with the percentages that represented each disease in the high and medium risk from the study group, thus obtaining the proportion of deaths directly caused by COVID-19 in these particular categories. The results

TABLE 8 | Relative risk, odds ratio, Pearson's Chi-Squared test for the most severe comorbidity.

Most severe comorbidity	Relative risk			*p*-value	Odds ratio			*p*-value	Pearson's Chi-Squared test
	Value	95% CI			Value	95% CI			
		Lower	Upper			Lower	Upper		
Diabetes	6.426	4.965	8.318	<0.05	6.820	5.173	8.990	<0.05	<0.05
Chronic renal disease	4.338	3.556	5.292	<0.05	4.742	3.796	5.925	<0.05	<0.05
Hypertension	3.261	2.687	3.958	<0.05	3.549	2.854	4.414	<0.05	<0.05
Chronic pulmonary disease	2.615	2.061	3.319	<0.05	2.751	2.125	3.561	<0.05	<0.05
Chronic liver disease	1.577	1.183	2.104	<0.05	1.610	1.188	2.184	<0.05	<0.05
Coronary heart disease	0.664	0.581	0.758	<0.05	0.574	0.485	0.680	<0.05	<0.05
Cancer	0.515	0.416	0.637	<0.05	0.464	0.367	0.587	<0.05	<0.05
Stroke	0.468	0.370	0.593	<0.05	0.423	0.328	0.546	<0.05	<0.05

TABLE 9 | COVID-19 influence on comorbidities.

Most severe comorbidity	Freq.	study group	Rom. Pop.	Relative risk (95% CI)	Attributable risk (95% CI)	Attributable risk % (95% CI)	Mean age (Std. Dev.)
COVID-19 high impact	**235**	**28.9%**	**7.0%**	**4.119** (3.694–4.592)	**0.219** (0.201–0.236)	**75.7%** (69.6%–81.8%)	**66.0** (13.32)
Diabetes	55	6.8%	1.1%	6.426 (4.965–8.318)	0.057 (0.05–0.064)	84.4% (74.0%–94.9%)	64.5 (11.54)
Chronic renal disease	88	10.8%	2.5%	4.338 (3.556–5.292)	0.083 (0.072–0.094)	76.9% (67.0%–86.9%)	68.1 (13.47)
Hypertension	92	11.3%	3.5%	3.261 (2.687–3.958)	0.078 (0.066–0.091)	69.3% (58.2%–80.5%)	65.0 (13.82)
COVID-19 medium impact	**107**	**13.1%**	**6.4%**	**2.058** (1.724–2.457)	**0.068** (0.051–0.084)	**51.4%** (38.6%–64.2%)	**64.8** (12.92)
Chronic pulmonary disease	63	7.7%	3.0%	2.615 (2.061–3.319)	0.048 (0.036–0.059)	61.8% (46.7%–76.9%)	67.4 (12.02)
Chronic liver disease	44	5.4%	3.4%	1.577 (1.183–2.104)	0.020 (0.036–0.059)	36.6% (13.4%–59.8%)	61.2 (13.42)
COVID-19 low impact	**312**	**38.3%**	**66.8%**	**0.574** (0.526–0.626)	**−0.285** (−0.317- −0.253)	**−74.3%** (−82.8%- −65.9%)	**72.0** (12.92)
Coronary heart disease	171	21.0%	31.7%	0.664 (0.581–0.758)	−0.107 (−0.139- −0.075)	−50.7% (−65.9%- −35.5%)	73.0 (12.82)
Cancer	77	9.5%	18.4%	0.515 (0.416–0.637)	−0.089 (−0.116- −0.063)	−94.3% (−122.5%- −66.1%)	68.6 (10.15)
Stroke	64	7.9%	16.8%	0.468 (0.370–0.593)	−0.089 (−0.115- −0.064)	−113.5% (−146.1%- −80.8%)	73.4 (9.89)
No & other comorbidities	**160**	**19.7%**	**16.0%**	**–**	**–**	**–**	**66.6** (16.18)
No comorbidities*	21	2.6%	3.8%	–	–	100%	59.0 (17.56)
Other comorbidities**	139	17.1%	16.0%	–	–	–	67.7 (15.72)
Total study group	**814**	100.0%	100.0%	–	–	–	**68.3** (13.61)

**Assumed all deaths with no comorbidities were due to covid-19.*

***Other comorbidities were not statistically significant, so were removed.*

were 21.9% (95% CI, 20.1–23.6%) out of 28.9% for the high risk group, 6.8% (95% CI, 5.1–8.4%) out of 1–3.1% for the medium risk group and 2.6% out of 2.6% for the group with no comorbidities. This led to a total of 31.2% (95% CI, 27.8–34.7% out of 44.6%, proportion of deaths that could have been directly caused by the SARS-CoV-2 infection. Furthermore, even though the patients in the last category (38.3%) were considered low risk and were statistically more likely to die because of their underlying condition, more information would be required in order to make a precise assessment of the proportion that

could have been directly attributed to SARS-CoV-2 infection. Moreover, in the case of people with other comorbidities (17.1%), a separate study should be conducted, that would take into consideration all of the particularities of the diseases found in that group.

Clinical Implications

This study has shown that in Romania, the number and type of comorbidities had an important contribution to the outcome of SARS-CoV-2 infection. After taking into consideration all of the aspects and because this paper comes as an additional confirmation of prior studies, one of the most important recommendations for clinical practice would be to offer extra protection to people that have certain types of underlying conditions. Diabetes, end stage renal disease and hypertension were shown to be at high risk when targeted by the novel coronavirus, while chronic pulmonary diseases (COPD and asthma) and chronic liver diseases (in particular cirrhosis) were moderately impacted. All the patients affected by any of these afflictions and especially those at high risk should be closely monitored by their physicians and in the eventuality of SARS-CoV-2 infection, they should present to a hospital in order to be immediately tested. Furthermore, when possible, they should be sheltered and if that desiderate cannot be achieved, they should at least be informed about their situation and the additional precautions they should be taking to protect themselves. Nonetheless their underlying condition should also be monitored and it should be kept under control with an adequate treatment.

In a study published in 2013, the outcome of 91,605 diabetic people was analyzed after the flu vaccination. Scientists then discovered a significant decrease, amongst these patients, in influenza and pneumonia incidence, of up to 43% for people under 65 years old and 55% for those over 65 (69). Taking into consideration the fact that COVID-19 has similar symptoms to influenza (fever, cough, and myalgia) and pneumonia, pneumococcal and flu vaccines could prove of great use in the colder seasons, not only for preventing these afflictions, but also for reducing the potential confusion between them. This matter could prove of great importance especially for people that are more at risk, such as those with the abovementioned comorbidities and the elderly. Lastly, the authors consider that the people in the high and medium risk groups should also be prioritized for future vaccination programs against SARS-CoV-2 in order to attempt to decrease mortality and hospitalization costs. This point is also supported by two official documents released by the WHO and the UK Government. Amongst the categories of people that are considered for vaccine prioritization in the two papers, those who are over 65 and/or suffering from diabetes, chronic kidney disease and chronic pulmonary disease have been included (70, 71).

Another important recommendation that emerged from this study would be for the people in the low risk category. They should be advised to respect their regular follow ups, according to their specialist and should be monitored for their underlying condition as usual. As the study has shown, for this category, the greatest risk is represented by their most severe illness, rather than the SARS-CoV-2 infection, in which case, missing doctors' appointments together with the natural progression of the disease could lead to a premature death. Amongst cancer patients, a recent study calculated an excess of 6,270 deaths in England and 33,890 in the USA, in 1 year due to missed or postponed medical appointments during the pandemic (72). For epidemiological research, the risk factors for each comorbidity could help model the potential fatality rates of SARS-CoV-2, within a given population, based upon the prevalence of these comorbidities.

Study Limitations

One of the limitations of this study was the lack of information about the people that have been infected and survived in Romania and as a result, the comparison between these two groups was not possible. However, the study was repurposed and the data was replaced with that of the deaths amongst the general population of Romania. This led to another constraint, given the fact that the most recent data available on the WHO website was from 2016. Furthermore, because of outbreaks in certain regions, some patients' information was incompletely reported, leading to a reduction in the study sample. Also, because this was a retrospective study and the data was collected from multiple sources, the accuracy of the information provided did not fall in the authors' responsibility. In addition to those mentioned above, the results discussed in this paper best reflect the population of Romania, as in other countries prevalence rates and therapeutic approaches may vary for the studied comorbidities and therefore, different outcomes could be observed.

Another limitation of this study was the lack of information regarding the initial clinical data at presentation, such as the level of hypoxia, the inflammatory markers and the severity of the disease, which could have offered a better insight on the impact of SARS-CoV-2 infection on the outcome.

Future Considerations

Another larger, ideally multicenter study comparing both the deceased and the survival group amongst those infected with SARS-CoV-2 would be beneficial in order to further identify preventable causes that led to a worse outcome. This study should also include details regarding laboratory parameters as well as radiological information. Additional analysis on the hospitalization rates for each comorbidity could help hospital capacity planning and preventing severe consequences. Moreover, future research should focus their efforts on developing therapeutic protocols that would improve the survival rates or vaccines to prevent infection.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: http://www.ms.ro/comunicate/.

AUTHOR CONTRIBUTIONS

MB, RT, and DT: conceptualization. MB, RT, DT, and DC: methodology. RT and DT: investigation. NS: resources and funding acquisition. MB and DT: writing—original draft preparation. DC: review, editing, and supervision. DT: project administration. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

We would like to thank the medical teams in Romania for the care they provide to patients suffering from COVID-19 and also keeping accurate medical records for us to use in research. We would also like to thank the Romanian Ministry of Health for making this data publicly available, free of charge.

REFERENCES

1. Wormser GP, Aitken C. Clinical virology, 3rd Edition. In: Richman DD, Whitley RJ, and Hayden FG, editors. *Clinical Infectious Diseases.* Washington, DC: ASM Press (2010) 50:1692.
2. Ksiazek TG, Erdman D, Goldsmith CS, Zaki SR, Peret T, Emery S, et al. A novel coronavirus associated with severe acute respiratory syndrome. *N Engl J Med.* (2003) 348:1953–66. doi: 10.1056/NEJMoa030781
3. de Groot RJ, Baker SC, Baric RS, Brown CS, Drosten C, Enjuanes L, et al. Middle east respiratory syndrome coronavirus (MERS-CoV): announcement of the coronavirus study group. *J Virol.* (2013) 87:7790–2. doi: 10.1128/JVI.01244-13
4. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet.* (2020) 395:497–506. doi: 10.1016/S0140-6736(20)30183-5
5. Zhu N, Zhang D, Wang W, Li X, Yang B, Song J, et al. A novel coronavirus from patients with pneumonia in China, (2019). *N Engl J Med.* (2020) 382:727–33. doi: 10.1056/NEJMoa2001017
6. Dawood AA. Mutated COVID-19 may foretell a great risk for mankind in the future. *New Microbes New Infect.* (2020) 35:100673. doi: 10.1016/j.nmni.2020.100673
7. Guan WJ, Ni ZY, Hu Y, Liang WH, Ou CQ, He JX, et al. Clinical characteristics of coronavirus disease 2019 in China. *N Eng J Med.* (2020) 382:1708–20. doi: 10.1056/NEJMoa2002032
8. Worldometers. *Coronavirus Worldwide Graphs*. (2020). Available online at: https://www.worldometers.info/coronavirus/worldwide-graphs/?fbclid=IwAR3y7JhX2P7Si1x8hwwaSBJHVepxa0apOnfDc72-lf7WZFEQTK6vrw1f570.%27%27 (accessed May 16, 2020).
9. Grupul de Informare Strategica. *Informare COVID−19, Grupul de Comunicare Strategică, 8 mai.* (2020). Available from: https://www.mai.gov.ro/21402-2/ (accessed May 8, 2020).
10. Spiegelhalter D. *How Much 'Normal' Risk Does Covid Represent?* (2020). Available online at: https://medium.com/wintoncentre/how-much-normal-risk-does-covid-represent-4539118e1196 (accessed May 10, 2020).
11. Yang J, Zheng Y, Gou X, Pu K, Chen Z, Guo Q, et al. Prevalence of comorbidities and its effects in coronavirus disease 2019 patients: a systematic review and meta-analysis. *Int J Infect Dis.* (2020) 94:91–5. doi: 10.1016/j.ijid.2020.03.017
12. Guan WJ, Liang WH, Zhao Y, Liang HR, Chen ZS, Li YM, et al. Comorbidity and its impact on 1590 patients with Covid-19 in China: a nationwide analysis. *Eur Respir J.* (2020) 55:2000547. doi: 10.1183/13993003.01227-2020
13. Statistics RNIf. *Homes and Population Census*. (2011). Available from: http://www.recensamantromania.ro/wp-content/uploads/2013/07/REZULTATE-DEFINITIVE-RPL_2011.pdf (accessed August 8, 2020)
14. Legislative Portal. *Specific Protocol From 13th March, 2020 Regarding the Management of the Deceased Patients who Were Infected With the Novel Coronavirus (SARS-CoV2)*. (2020). Available online at: http://legislatie.just.ro/Public/DetaliiDocument/223812?fbclid=IwAR03roBO_iSyatI4H_FrBj_l6_4TChh_9-asAulZ8Z1tIXtwlMtFR5iDMSA (accessed May 5, 2020).
15. Legislative Portal. *Order no. 570 From 6th April, 2020 to Modifiy the Order of the Minister of Health, no. 436/2020, for the Approval of the Protocol Regarding the Management of the Deceased Patients who Were Infected With the Novel Coronavirus (SARS-CoV2).* (2020). Available online at: http://legislatie.just.ro/Public/DetaliiDocumentAfis/224715 (accessed May 5, 2020).
16. Organization WH. *International Guidelines for Certification and Classification (Coding) of Covid-19 as Cause of Death*. (2020). Available online at: https://www.who.int/classifications/icd/Guidelines_Cause_of_Death_COVID-19.pdf?ua=1 (accessed August 7, 2020).
17. Liu TZ, Zhang DS, Liang Y, Zhou NN, Gao HF, Liu KJ, et al. Treatment strategies and prognostic factors of patients with primary germ cell tumors in the mediastinum. *J Cancer Res Clin Oncol.* (2011) 137:1607–12. doi: 10.1007/s00432-011-1028-7
18. Pancreatic Cancer Action Network. *Pancreatic Cancer Survival Rates*. (2020). Available online at: https://www.pancan.org/facing-pancreatic-cancer/about-pancreatic-cancer/survival-rate/ (accessed May 6, 2020).
19. American Cancer Society. *Lung Cancer Survival Rates*. (2020). Available online at: https://www.cancer.org/cancer/lung-cancer/detection-diagnosis-staging/survival-rates.html (accessed May 6, 2020).
20. Cancer Research UK. *Acute Lymphoblastic Leukemia – Survival*. (2020). Available online at: https://www.cancerresearchuk.org/about-cancer/acute-lymphoblastic-leukaemia-all/survival (accessed May 6, 2020).
21. American Cancer Society. *Survival Rates for Colorectal Cancer*. (2020). Available online at: https://www.cancer.org/cancer/colon-rectal-cancer/detection-diagnosis-staging/survival-rates.html (accessed May 8, 2020).
22. Ovarian Cancer Research Alliance. *About Ovarian Cancer – Staging*. (2020). Available from: https://ocrahope.org/patients/about-ovarian-cancer/staging/ (accessed May 6, 2020).
23. American Cancer Society. *Liver Cancer Survival Rates*. (2020). Available online at: https://www.cancer.org/cancer/liver-cancer/detection-diagnosis-staging/survival-rates.html (accessed May 8, 2020).
24. GynaeHealthUK. *Cervical Cancer Prognosis and Survival Rates*. (2020). Available online at: https://www.gynaehealthuk.com/cervical-cancer-information/cervical-cancer-prognosis-and-survival-rates (accessed May 6, 2020).
25. BreastCancer.org. *What Does Prognosis Mean?* (2017). Available online at: https://www.breastcancer.org/symptoms/diagnosis/prognosis (accessed May 5, 2020).
26. American Cancer Society. *Survival Rates for Melanoma Skin Cancer*. (2020). Available online at: https://www.cancer.org/cancer/melanoma-skin-cancer/detection-diagnosis-staging/survival-rates-for-melanoma-skin-cancer-by-stage.html (accessed May 6, 2020).
27. American Cancer Society. *Stomach Cancer Survival Rates*. (2020). Available online at: https://www.cancer.org/cancer/stomach-cancer/detection-diagnosis-staging/survival-rates.html (accessed May 7, 2020).
28. American Cancer Society. *Survival Rates for Waldenstrom Macroglobulinemia*. (2018). Available online at: https://www.cancer.org/cancer/waldenstrom-macroglobulinemia/detection-diagnosis-staging/survival-rates.html (accessed May 8, 2020).
29. Man D, Wu J, Shen Z, Zhu X. Prognosis of patients with neuroendocrine tumor: a SEER database analysis. *Cancer Manag Res.* (2018) 10:5629–38. doi: 10.2147/CMAR.S174907
30. American Cancer Society. *Survival Rates for Laryngeal and Hypopharyngeal Cancers*. (2020). Available online at: https://www.cancer.org/cancer/laryngeal-and-hypopharyngeal-cancer/detection-diagnosis-staging/survival-rates.html (accessed May 8, 2020).
31. Arora B, Sirhan S, Hoyer JD, Mesa RA, Tefferi A. Peripheral blood CD34 count in myelofibrosis with myeloid metaplasia: a prospective evaluation of prognostic value in 94 patients. *Br J Haematol.* (2005) 128:42–8. doi: 10.1111/j.1365-2141.2004.05280.x

32. Cancer.NET. *Multiple Myeloma: Statistics*. (2020). Available online at: https://www.cancer.net/cancer-types/multiple-myeloma/statistics (accessed May 8, 2020).
33. McCarthy BJ, Davis FG, Freels S, Surawicz TS, Damek DM, Grutsch J, et al. Factors associated with survival in patients with meningioma. *J Neurosurg.* (1998) 88:831–9. doi: 10.3171/jns.1998.88.5.0831
34. Cancer.NET. *Oral and Oropharyngeal Cancer: Statistics*. (2020). Available online at: https://www.cancer.net/cancer-types/oral-and-oropharyngeal-cancer/statistics (accessed May 6, 2020).
35. American Cancer Society. *Survival Rates for Small Intestine Cancer (Adenocarcinoma)*. (2020). Available online at: https://www.cancer.org/cancer/small-intestine-cancer/detection-diagnosis-staging/survival-rates.html (accessed May 8, 2020).
36. Pulte D, Castro FA, Jansen L, Luttmann S, Holleczek B, Nennecke A, et al. Trends in survival of chronic lymphocytic leukemia patients in Germany and the USA in the first decade of the twenty-first century. *J Hematol Oncol.* (2016) 9:28. doi: 10.1186/s13045-016-0257-2
37. Gottwald L, Pluta P, Piekarski J, Spych M, Hendzel K, Topczewska-Tylinska K, et al. Long-term survival of endometrioid endometrial cancer patients. *Arch Med Sci.* (2010) 6:937–44. doi: 10.5114/aoms.2010.19305
38. American Cancer Society. *Survival Rates for Prostate Cancer*. (2020). Available online at: https://www.cancer.org/cancer/prostate-cancer/detection-diagnosis-staging/survival-rates.html (accessed May 8, 2020).
39. Ratib S, Fleming KM, Crooks CJ, Aithal GP, West J. 1 and 5 year survival estimates for people with cirrhosis of the liver in England, 1998-2009: a large population study. *J Hepatol.* (2014). 60:282–9. doi: 10.1016/j.jhep.2013.09.027
40. Chung LP, Winship P, Phung S, Lake F, Waterer G. Five-year outcome in COPD patients after their first episode of acute exacerbation treated with non-invasive ventilation. *Respirology.* (2010) 15:1084–91. doi: 10.1111/j.1440-1843.2010.01795.x
41. Simpson CR, Buckley BS, McLernon DJ, Sheikh A, Murphy A, Hannaford PC. Five-year prognosis in an incident cohort of people presenting with acute myocardial infarction. *PLOS ONE.* (2011) 6:e26573. doi: 10.1371/journal.pone.0026573
42. Sun Y, Lee SH, Heng BH, Chin VS. 5-year survival and rehospitalization due to stroke recurrence among patients with hemorrhagic or ischemic strokes in Singapore. *BMC Neurology.* (2013). 13:133. doi: 10.1186/1471-2377-13-133
43. University of California San Francisco. *The Kidney Project*. (2018). Available online at: https://pharm.ucsf.edu/kidney/need/statistics (accessed 8 May, 2020).
44. Organization WH. *Cardiovascular Diseases*. (2020). Available online at: https://www.who.int/health-topics/cardiovascular-diseases/#tab=tab_1 (accessed May 5, 2020).
45. Nowbar AN, Gitto M, Howard JP, Francis DP, Al-Lamee R. Mortality from ischemic heart disease. *Circ Cardiovasc Qual Outcomes.* (2019) 12:e005375. doi: 10.1161/CIRCOUTCOMES.118.005375
46. World Health Organization. *European Health for All Database (HFA-DB)*. (2019). Available online at: https://gateway.euro.who.int/en/datasets/european-health-for-all-database/ (accessed May 4, 2020).
47. World Health Organization. *Disease Burden and Mortality Estimates*. (2020). Available online at: https://www.who.int/healthinfo/global_burden_disease/estimates/en/ (accessed May 4, 2020).
48. Global Initiative for Chronic Obstructive Lung Disease. *World Lung Day 2019: Respiratory Groups Unite to Call for Healthy Lungs for All*. (2020). Available online at: https://goldcopd.org/world-lung-day-2019-respiratory-groups-unite-to-call-for-healthy-lungs-for-all/ (accessed May 7, 2020).
49. Tripathi A. *What is the Coefficient of Determination | R Square*. (2020). Available online at: https://ashutoshtripathi.com/2019/01/22/what-is-the-coefficient-of-determination-r-square/ (accessed 12 May, 2020).
50. Meng Y, Wu P, Lu W, Liu K, Ma K, Huang L, et al. Sex-specific clinical characteristics and prognosis of coronavirus disease-19 infection in Wuhan, China: a retrospective study of 168 severe patients. *PLOS Pathogens.* (2020) 16:e1008520. doi: 10.1371/journal.ppat.1008520
51. Badawi A, Ryoo SG. Prevalence of comorbidities in the Middle East respiratory syndrome coronavirus (MERS-CoV): a systematic review and meta-analysis. *Int J Infect Dis.* (2016) 49:129–33. doi: 10.1016/j.ijid.2016.06.015
52. Karlberg J, Chong DSY, Lai WYY. Do men have a higher case fatality rate of severe acute respiratory syndrome than women do? *Am J Epidemiol.* (2004) 159:229–31. doi: 10.1093/aje/kwh056
53. Klein SL, Flanagan KL. Sex differences in immune responses. *Nat Rev Immunol.* (2016) 16:626–38. doi: 10.1038/nri.2016.90
54. Pinheiro I, Dejager L, Libert C. X-chromosome-located microRNAs in immunity: might they explain male/female differences? The X chromosome-genomic context may affect X-located miRNAs and downstream signaling, thereby contributing to the enhanced immune response of females. *Bioessays.* (2011) 33:791–802. doi: 10.1002/bies.201100047
55. Wang Y, Hunt K, Nazareth I, Freemantle N, Petersen I. Do men consult less than women? An analysis of routinely collected UK general practice data. *BMJ Open.* (2013) 3:e003320. doi: 10.1136/bmjopen-2013-003320
56. Zhonghua L, Xing B, Xue ZZ. The epidemiological characteristics of an outbreak of 2019 novel coronavirus diseases (COVID-19) in China. *Zhonghua Liu Xing Bing Xue Za Zhi.* (2020) 41:145–51. doi: 10.3760/cma.j.issn.0254-6450.2020.02.003
57. Zhou F, Yu T, Du R, Fan G, Liu Y, Liu Z, et al. Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: a retrospective cohort study. *Lancet.* (2020) 395:1054–62. doi: 10.1016/S0140-6736(20)30566-3
58. Kumar A, Arora A, Sharma P, Anikhindi SA, Bansal N, Singla V, et al. Is diabetes mellitus associated with mortality and severity of COVID-19? A meta-analysis. *Diabetes Metab Syndr Clin Res Rev.* (2020) 14:535–45. doi: 10.1016/j.dsx.2020.04.044
59. Huang I, Lim MA, Pranata R. Diabetes mellitus is associated with increased mortality and severity of disease in COVID-19 pneumonia – a systematic review, meta-analysis, and meta-regression. *Diabetes Metab Syndr Clin Res Rev.* (2020) 14:395–403. doi: 10.1016/j.dsx.2020.04.018
60. Cheng Y, Luo R, Wang K, Zhang M, Wang Z, Dong L, et al. Kidney disease is associated with in-hospital death of patients with COVID-19. *Kidney Int.* (2020) 97:829–38. doi: 10.1016/j.kint.2020.03.005
61. Henry BM, Lippi G. Chronic kidney disease is associated with severe coronavirus disease (2019). (COVID-19) infection. *Int Urol Nephrol.* (2020). 52:1193–4. doi: 10.1007/s11255-020-02451-9
62. Wang D, Hu B, Hu C, Zhu F, Liu X, Zhang J, et al. Clinical Characteristics of 138 Hospitalized Patients With (2019). Novel Coronavirus-Infected Pneumonia in Wuhan, China. *JAMA.* (2020) 323:1061–9. doi: 10.1001/jama.2020.1585
63. Zhang JJ, Dong X, Cao YY, Yuan YD, Yang YB, Yan YQ, et al. Clinical characteristics of 140 patients infected with SARS-CoV-2 in Wuhan, China. *Allergy.* (2020) 75:1730–41. doi: 10.1111/all.14238
64. Zuin M, Rigatelli G, Zuliani G, Rigatelli A, Mazza A, Roncon L. Arterial hypertension and risk of death in patients with COVID-19 infection: systematic review and meta-analysis. *J Infect.* (2020) 81:e84–e6. doi: 10.1016/j.jinf.2020.03.059
65. Lippi G, Wong J, Henry B. Hypertension and its severity or mortality in coronavirus Disease (2019). (COVID-19): a pooled analysis. *Pol Arch Medycyny Wewn?trznej.* (2020). 130:304–9. doi: 10.20452/pamw.15272
66. Perazzo H, Pacheco AG, De Boni R, Luz PM, Fittipaldi J, Cardoso SW, et al. Age-standardized mortality rates related to cirrhosis in brazil from 2000 to 2012: a nationwide analysis. *Ann Hepatol.* (2017) 16:269–78. doi: 10.5604/16652681.1231588
67. Fang L, Karakiulakis G, Roth M. Are patients with hypertension and diabetes mellitus at increased risk for COVID-19 infection? *Lancet Res Med.* (2020) 8:e21. doi: 10.1016/S2213-2600(20)30116-8
68. Soler MJ, Wysocki J, Batlle D. ACE2 alterations in kidney disease. *Nephrol Dial Transplant.* (2013) 28:2687–97. doi: 10.1093/ndt/gft320

69. Lau D, Eurich D, Majumdar S, Katz A, Johnson J. Effectiveness of influenza vaccination in working-age adults with diabetes: a population-based cohort study. *Thorax*. (2013) 68:658–63. doi: 10.1136/thoraxjnl-2012-203109
70. Organization WH. *A Global Framework to Ensure Equitable and Fair Allocation of Covid-19 Products*. (2020). Available online at: https://apps.who.int/gb/COVID-19/pdf_files/18_06/Global%20Allocation%20Framework.pdf?utm_source=POLITICO.EU&utm_campaign=18fd118248-EMAIL_CAMPAIGN_2020_06_22_04_52_COPY_01&utm_medium=email&utm_term=0_10959edeb5-18fd118248-189787901 (accessed August 8, 2020).
71. Care UDoHaS. *Joint Committee on Vaccination and Immunisation: Interim Advice on Priority Groups for COVID-19 Vaccination*. (2020). Available online at: https://www.gov.uk/government/publications/priority-groups-for-coronavirus-covid-19-vaccination-advice-from-the-jcvi/interim-advice-on-priority-groups-for-covid-19-vaccination (accessed August 8, 2020).
72. Lai A, Pasea L, Banerjee A, Denaxas S, Katsoulis M, Chang WH, et al. Estimating excess mortality in people with cancer and multimorbidity in the COVID-19 emergency. *medRxiv*. (2020). doi: 10.1101/2020.05.27.20083287

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Public Health

OPINION
published: 09 September 2020
doi: 10.3389/fpubh.2020.00490

Thoughts From the Trenches: Should We Look at the "Healthy"?

Víctor M. Martínez-Taboada[1,2]*, *Marcos López-Hoyos*[3], *Javier Crespo*[2,4], *Pedro Muñoz Cacho*[5] and *José L. Hernández*[2,6]

[1] Division of Rheumatology, Hospital Marqués de Valdecilla-IDIVAL, Santander, Spain, [2] University of Cantabria, Santander, Spain, [3] Division of Immunology, Hospital Marqués de Valdecilla-IDIVAL, Santander, Spain, [4] Division of Gastroenterology, Hospital Marqués de Valdecilla-IDIVAL, Santander, Spain, [5] Department of Medicine and Psychiatry, Gerencia de Atención Primaria, Servicio Cántabro de Salud, Santander, Spain, [6] Department of Internal Medicine, Hospital Marqués de Valdecilla-IDIVAL, Santander, Spain

Keywords: SARS-CoV-2 (virus), Spain, serological tests, research, COVID-19

INTRODUCTION

The pandemic triggered after the massive spread of SARS-CoV-2 from Wuhan (1) is hitting most countries with varying degrees of virulence, striking particularly hard in Spain. A pandemic without precedent, and of a magnitude and severity unknown to this century. Let's contrast and compare some recent data on other seasonal respiratory viruses with serious health consequences. During the 2018–2019 influenza epidemic in Spain, there were 490,000 non-serious cases, 35,300 hospitalizations, 2,500 patients were admitted to the intensive care units (ICU) and 6,300 died (https://vacunasaep.org). Concerning SARS-CoV-2 infection, on May 15, 2020, the Spanish Ministry of Health (https://www.mscbs.gob.es) had reported 230,183 cases, 124,571 hospitalizations, 11,493 ICU admissions, and 27,459 deaths. Therefore, the numbers speak for themselves when both infections were compared.

It is true that the Spanish Healthcare System has some peculiarities that may have made initial control of the pandemic difficult. These include the system fragmentation into 17 health regions and the absence of a proactive strategy to tracing contacts or search for potential cases, coupled with an absence of preventive measures to foresee the supply chain shortages for personal protection equipment and diagnostics tests (RNA extraction reagents and RT-PCR kits). Nevertheless, the Spanish Healthcare System has demonstrated great flexibility and adaptability during pandemics. In fact, hospital beds and intensive care facilities have increased, even external hospitalization centers such as the IFEMA in Madrid with up to 5,000 beds (an ∼39% increase in the number of hospital beds in one of the most affected regions in Spain) have been set up in record time to attend COVID-19 patients, and a number of research laboratories (24 laboratories accreditated by the National Research Institute Carlos III) got ready to do RT-PCR diagnostics test across the country. The Spanish civil society has also demonstrated a great responsibility in adopting all the preventive measures taken by the health authorities. Besides, our knowledge of the best therapeutic measures to fight against coronavirus is rapidly increasing. Therefore, there is likely to be room for hope even in the case of a second SARS-CoV-2 wave in our country.

Without a doubt, the economic impact in a system highly dependent on the tourism and service sectors will be enormous, with the consequent impoverishment and added difficulty in taking public health actions. At the time of writing this manuscript, and with the confinement period just finished, the Spanish government, advised by experts, has implemented a number of measures intended to gradually start the normal daily activities. These actions are accompanied by increased SARS-CoV-2 testing to know the degree of immunization in the general population (2). However, within the first weeks of "*normal activity*" we are seeing an ever increasing number of traceable outbreaks and, in some cases, they are at risk of developing as community epidemics in a second wave.

OPEN ACCESS

Edited by:
Zisis Kozlakidis, International Agency For Research On Cancer (IARC), France

Reviewed by:
Pier Luigi Meroni, Istituto Auxologico Italiano (IRCCS), Italy
Rocio Aguilar-Quesada, Andalusian Public Health System Biobank, Spain

***Correspondence:**
Víctor M. Martínez-Taboada
vmartinezt64@gmail.com

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Public Health

Received: *22 May 2020*
Accepted: *31 July 2020*
Published: *09 September 2020*

Citation:
Martínez-Taboada VM, López-Hoyos M, Crespo J, Cacho PM and Hernández JL (2020) Thoughts From the Trenches: Should We Look at the "Healthy"? Front. Public Health 8:490. doi: 10.3389/fpubh.2020.00490

WILL THE SANITARY PASSPORT CLEAR ALL OUR DOUBTS?

Due to several reasons, we deem that classifying the individuals into immunized and non-immunized groups will not solve all our troubles as most people believe:

(a) At present, not only do we not know the real prevalence of the viral infection, but more importantly, we do not know what the progression of the pandemic will be. We also do not know whether subjects who have had COVID19 will be immune in a possible future SARS-CoV-2 wave, probably next winter (3). Besides, we still do not appreciate the mutation capacity of SARS-CoV-2 and its impact on infectivity and lethality. Preliminary data from Spain big survey on seroprevalence indicates 5.2% overall humoral immunity (4).

(b) There are some doubts about the sensitivity and specificity of SARS-CoV-2 serological tests, as well as about the SARS-CoV-2 epitopes they include. Thus, these tests should be interpreted with caution, especially in the case of lateral flow rapid tests (5).

(c) Although the original intention is on massive population-wide testing, the impossibility of carrying out this measure in the entire Spanish population is clear. From the outset, it seems reasonable to select the high-risk groups for testing: elderly people (especially nursing homes residents), immunosuppressed patients, health care workers, etc. (6). A different issue arises when a low-risk population is considered: Are we going to force the entire population to test? Are we going to test all the children or youth people? In fact, although there is little information (7) they are probably the main asymptomatic groups and potentially spreaders of the disease to high-risk individuals. Besides, some experts consider that summer is a season with a lower incidence of viral infections not only because of climatic factors but also because, with schools closed, viruses have much less ability to spread, as it has happened with other viral outbreaks.

(d) Therefore, not only are there factors dependent on the virus, but we also ought to take into account host factors. Not everyone may be capable of developing an effective immune response against the infection. It is also unclear what level of antibodies one should reach, or whether this level could protect against a second encounter with the virus or any of its variants (8). If the outbreak becomes endemic-seasonal, the possibility of response might also decrease over time. On the other hand, recent evidences point to the protective role of cellular immunity, which has not been taken into account until very recently, either cross-reactive with seasonal coronavirus (9) or after SARS-CoV-2 infection (10).

(e) Something very similar would happen if we hopefully had an effective vaccine. It might not protect everyone, or at least not equally. Furthermore, the duration of a proper immunization is also unknown. Therefore, until we increase our knowledge about immunity generated by SARS-CoV2, continuing to work on general preventive actions and antiviral treatments seems to be the key to success against this infection. The use of facial mask and hand washing, together with social distancing, will remain the main actions in counteracting the pandemic.

DISCUSSION

Given the current uncertainty derived from the lack of knowledge of the natural history of the viral disease, we can anticipate two extreme scenarios:

1) Favorable scenario: a virus with little ability to mutate, the development of permanent humoral and cellular immunity, adequate preventive actions (including general measures, effective pharmacological interventions, and vaccines), the discovery of effective antiviral drugs, and no appearance of another breakthrough pandemic.
2) Unfavorable scenario: a virus with high capacity for mutation, the development of transient immunity, inadequate preventive measures, the lack of effective antiviral agents, or a new pandemic breaking out.

It is crucial to bear in mind that it is extremely complicated for all the favorable conditions to eradicate COVID19. Nonetheless, the presence of a single unfavorable condition can be critical in the general outcome of the pandemic.

So far in Spain, only those patients with moderate-severe symptoms have been tested for coronavirus with PCR tests. The health system has made a heroic effort to treat those patients with serious COVID19, and several drugs have been used with a rational pathophysiological principle, despite the lack of solid scientific evidence to date (11). On the other hand, not only in Spain but in the rest of the world, numerous well-designed studies have been launched in a short period of time, to demonstrate whether the different therapeutic agents we are currently using can really be effective in treating COVID19. It stands to reason that knowing the real efficacy of these therapeutic agents will help patients who become infected in the future to overcome their disease and design treatment protocols according to its severity, although today none of these agents seems to have a curative role. Only the collective effort of the international scientific community, through the careful study of SARS-CoV-2 characteristics, the intimate pathogenic mechanisms of the disease, the pattern of the different responses of the host to infection, and the protective health measures against it, would help to control this pandemic promptly.

The possibilities that we are going to face and the different clinical scenarios in which we have to fight to overcome COVID19 are shown in the **Table 1**. The war against this serious disease, as previously discussed, is still being waged without truce. This fight will provide relevant information for new patients with severe COVID19 manifestations: identifying prognostic factors for disease severity and progression to ARDS (12, 13), and determine the real efficacy of therapeutic options through well-designed clinical trials (14). The study of mild cases of the disease and the asymptomatic carriers might help us answer other relevant questions, such as the duration of the contagious period, the characteristics of the non-severe disease or even the type, intensity and duration of immunization (8). The issue of immunization is relevant in the development of vaccination strategies once they are ready to be used.

A critical part of the fight against SARS-CoV-2 is the improvement in diagnostic tests, not only during the acute

TABLE 1 | Possible scenarios, groups susceptible to intervention, and opportunities in SARS-CoV-2 infection.

Individuals		Scenario	Susceptible to intervention	Opportunities
INFECTED	- Clinical* +/Test +	- Serious disease. Hospital admission	No	- Study available and new treatment options (randomized controlled trials) - Identify prognostic factors for disease severity and progression to ARDS
		- Mild disease. Outpatient treatment		- Determine the characteristics for non-severe disease - Determine contagious period - Determine duration of immunization
	- Clinical +/Test -	- False negative test result	Yes	- Improve molecular diagnostic tests (conventional RT-PCR, Xpert RT-PCR, LAMP)
		- COVID19 ruled out (others URTI)		- Identify (**) and prophylaxis (?)
	- Clinical +/Test ND	- Mild disease. Outpatient control	Yes	- Identify and determine the characteristics for non-severe disease
		- COVID19 ruled out (others URTI)		- Identify and prophylaxis (?)
	- Clinical—/ Test ND	- Asymptomatic carriers	No	- Identify and determine characteristics for non-severe disease - Determine the duration of immunization
"SUPPOSEDLY NOT INFECTED"	- Clinical—/Test ND	- COVID19 contact +/No high-risk group	Yes	- Identify and determine immune status - Prophylaxis accordingly
	- Clinical—/Test ND	- COVID19 contact+/High-risk group *(elderly, immunosuppressed patients, heath care workers, etc.)*	Yes	- Identify and determine immune status - IgM—/ IgG—(mandatory prophylaxis)
	- Clinical—/Test ND	- No contact/No high risk group *They are impossible to distinguish from asymptomatic carriers*	Yes (?)	- Identify and determine immune status (?) -Prophylaxis accordingly (?)
	- Clinical—/Test ND	- No contact/High-risk group *(elderly, immunosuppressed patients, health care workers, etc.)*	Yes	- Identify and determine immune status - IgM—/IgG—(mandatory prophylaxis)

**Clinical, clinical manifestations suggestive of respiratory tract infection of any cause; Test, means RT-PCR; ND, not done; URTI, upper respiratory tract infection; ARDS, acute respiratory distress syndrome; RT-PCR, real time quantitative polymerase chain reaction; LAMP, loop-mediated isothermal amplification). (**) Identify, means to determine immunization status through reliable serological tests.*

phase of the disease, but also in the sensitivity and specificity of serological tests (6, 15). Furthermore, the development of more suitable tests to determine the cellular immunity against the virus, will improve the knowledge of the disease and the opportunities for intervention. This is a key aspect, especially in high risk groups, where in the absence of curative treatments for the infection, establishing appropriate prophylaxis measures is crucial. Nowadays, and pending the results of appropriate studies (antimalarial drugs, vitamin D, trained immunity, etc.) (16–19), the only preventive measures that have been proven effective are the implementation of precautions and hygienic measures to minimize human transmission of the virus, especially in high-risk populations.

The investigation focused on "healthy" people who have contacted the virus and did not develop the disease, or those who have been cured without problems (including the asymptomatic carriers) is a crucial issue (9). Probably, this may be the key to identifying new therapeutic or preventive interventions. Perhaps, we ought to go for strategies that combine the study of the "sick" and the "healthy" individual. In the meantime, it is probably utopian to reach the entire population with an appropriate diagnostic test, but we must prepare ourselves to reach the maximum number of people, with a logistical and economic effort of unprecedented dimensions. At this moment, probably the most cost-effective approach will rely in the urgent identification of cases and outbreaks by RT-PCR together with searching of contacts to trace any focus (20). However, this will all be investment and not spending. As it is evident, we have a lot of work to do but, at the same time, a lot of opportunities to explore.

AUTHOR CONTRIBUTIONS

VM-T designed the manuscript. VM-T, JH, ML-H, JC, and PC wrote the manuscript. All authors discussed the results and contributed to the final paper.

REFERENCES

1. Zhu N, Zhang D, Wang W, Li X, Yang B, Song J, et al. A novel coronavirus from patients with pneumonia in China, 2019. *N Engl J Med.* (2020) 382:727–33. doi: 10.1056/NEJMoa2001017
2. Fineberg HV. Ten weeks to crush the curve. *N Engl J Med.* (2020) 382:e37. doi: 10.1056/NEJMe2007263
3. Neher RA, Dyrdak R, Druelle V, Hodcroft EB, Albert J. Potential impact of seasonal forcing on a SARS-CoV-2 pandemic. *Swiss Med Wkly.* (2020) 150:w20224. doi: 10.4414/smw.2020.20224
4. Pollán M, Pérez-Gómez B, Pastor-Barriuso R, Oteo J, Hernán MA, Pérez-Olmeda M, et al. Prevalence of SARS-CoV-2 in Spain (ENE-COVID): a nationwide, population-based seroepidemiological study. *Lancet.* (2020) S0140-6736 (20)31486-5. doi: 10.1016/S0140-6736(20)31483-5
5. Petherick A. Developing antibody tests for SARS-CoV-2. *Lancet.* (2020) 395:1101–2. doi: 10.1016/S0140-6736(20)30788-1
6. Cheng MP, Papenburg J, Desjardins M, Kanjilal S, Quach C, Libman M, et al. Diagnostic testing for severe acute respiratory syndrome-related coronavirus-2: a narrative review. *Ann Intern Med.* 172:726–34. doi: 10.7326/M20-1301
7. L Gudbjartsson DF, Helgason A, Jonsson H, Magnusson OT, Melsted P, Norddahl GL, et al. Spread of SARS-CoV-2 in the icelandic population. *N Engl J Med.* (2020) 382:2302–15. doi: 10.1101/2020.03.26.20044446
8. Ibarrondo FJ, Fulcher JA, Goodman-Meza D, Elliott J, Hofmann C, Hausner MA, et al. Rapid decay of anti-SARS-CoV-2 antibodies in persons with mild covid-19 [published online ahead of print, (2020). *N Engl J Med.* (2020) NEJMc2025179. doi: 10.1056/NEJMc2025179
9. Grifoni A, Weiskopf D, Ramirez SI, Mateus J, Dan JM, Moderbacher CR, et al. Targets of T cell responses to SARS-CoV-2 coronavirus in humans with COVID-19 disease and unexposed individuals. *Cell.* (2020) 181:1489–501.e15. doi: 10.1016/j.cell.2020.05.015
10. Sekine T, Perez-Potti A, Rivera-Ballesteros O, Strï£¡lin K, Gorin JB, Olsson A, et al. Robust T cell immunity in convalescent individuals with asymptomatic or mild COVID-19. *Cell.* (2020) doi: 10.1016/j.cell.2020.08.017
11. Coronavirus Disease (COVID-19) Treatment Guidelines 2020 (2019). Available online at: https://covid19treatmentguidelines.nih.gov/ (accessed April 22 2020).
12. van Gerwen M, Alsen M, Little C, Barlow J, Genden E, Naymagon L, et al. Risk factors and outcomes of COVID-19 in New York City; a retrospective cohort study [published online ahead of print 2020. *J Med Virol.* (2020). doi: 10.1002/jmv.26337
13. Jesenak M, Brndiarova M, Urbancikova I, Rennerova Z, Vojtkova J, Bobcakova A, et al. Immune parameters and COVID-19 infection—associations with clinical severity and disease prognosis. *Front Cell Infect Microbiol.* (2020) 10:364. doi: 10.3389/fcimb.2020.00364
14. Olender SA, Perez KK, Go AS, Balani B, Price-Haywood EG, Shah NS, et al. Remdesivir for Severe COVID-19 versus a Cohort Receiving Standard of Care. *Clin Infect Dis.* (2020) ciaa1041. doi: 10.1093/cid/ciaa1041
15. D'Cruz RJ, Currier AW, Sampson VB. Laboratory testing methods for novel severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2). *Front Cell Dev Biol.* (2020) 8:468. doi: 10.3389/fcell.2020.00468
16. Ferreira A, Oliveira-E-Silva A, Bettencourt P. Chronic treatment with hydroxychloroquine and SARS-CoV-2 infection. *J Med Virol.* (2020) doi: 10.1101/2020.06.26.20056507. [Epub ahead of print].
17. Galvis V, Spinelli FR, Tello A, Sossa CL, Higuera JD, Gómez ED, et al. Hydroxychloroquine as prophylaxis for coronavirus SARS-CoV-2 infection: review of the ongoing clinical trials. *Arch Bronconeumol.* (2020) S0300-2896(20)30162-9. doi: 10.1016/j.arbres.2020.05.008
18. Arboleda JF, Urcuqui-Inchima S. Vitamin D supplementation: a potential approach for coronavirus/covid-19 therapeutics?. *Front Immunol.* (2020) 11:1523. doi: 10.3389/fimmu.2020.01523
19. Netea MG, Domínguez-Andrés J, Barreiro LB, Chavakis T, Divangahi M, Fuchs E, et al. Defining trained immunity and its role in health and disease. *Nat Rev Immunol.* (2020) 20:375–88. doi: 10.1038/s41577-020-0285-6
20. Allam M, Cai S, Ganesh S, Venkatesan M, Doodhwala S, Song Z, et al. COVID-19 diagnostics, tools, and prevention. *Diagnostics (Basel).* (2020) 10:409. doi: 10.3390/diagnostics10060409

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Public Health

PERSPECTIVE
published: 10 September 2020
doi: 10.3389/fpubh.2020.00513

A Basic Review of the Preliminary Evidence That COVID-19 Risk and Severity Is Increased in Vitamin D Deficiency

Linda L. Benskin [1,2]*

[1] Independent Researcher for Improving Health in Rural Areas of Tropical Developing Countries, Austin, TX, United States, [2] Ferris Mfg. Corp., Makers of PolyMem® Multifunctional Dressings, Ft. Worth, TX, United States

As the world's attention has been riveted upon the growing COVID-19 pandemic, many researchers have written brief reports supporting the hypothesis that vitamin D deficiency is related to the incidence and severity of COVID-19. The clear common thread among the top risk groups—vitamin D deficiency—may be being overlooked because of previous overstated claims of vitamin D benefits. However, the need to decrease COVID-19 fatalities among high-risk populations is urgent. Early researchers reported three striking patterns. Firstly, the innate immune system is impaired by vitamin D deficiency, which would predispose sufferers to viral infections such as COVID-19. Vitamin D deficiency also increases the activity of the X-chromosome-linked "Renin-Angiotensin" System, making vitamin D deficient individuals (especially men) more susceptible to COVID-19's deadly "cytokine storm" (dramatic immune system overreaction). Secondly, the groups who are at highest risk for severe COVID-19 match those who are at highest risk for severe vitamin D deficiency. This includes the elderly, men, ethnic groups whose skin is naturally rich in melanin (if living outside the tropics), those who avoid sun exposure for cultural and health reasons, those who live in institutions, the obese, and/or those who suffer with hypertension, cardiovascular disease, or diabetes. And thirdly, the pattern of geographical spread of COVID-19 reflects higher population vitamin D deficiency. Both within the USA and throughout the world, COVID-19 fatality rates parallel vitamin D deficiency rates. A literature search was performed on PubMed, Google Scholar, and RSMLDS, with targeted Google searches providing additional sources. Although randomized controlled trial results may be available eventually, the correlational and causal study evidence supporting a link between vitamin D deficiency and COVID-19 risks is already so strong that it supports action. The 141 author groups writing primarily about biological plausibility detailed how vitamin D deficiency can explain every risk factor and every complication of COVID-19, but agreed that other factors are undoubtedly at work. COVID-19 was compared with dengue fever, for which oral vitamin D supplements of 4,000 IU for 10 days were significantly more effective than 1,000 IU in reducing virus replication and controlling the "cytokine storm" (dramatic immune system over-reaction) responsible for fatalities. Among the 47 original research studies summarized here, chart reviews found that serum vitamin D levels predicted COVID-19 mortality

OPEN ACCESS

Edited by:
Zisis Kozlakidis, International Agency for Research on Cancer (IARC), France

Reviewed by:
Rimesh Pal, Post Graduate Institute of Medical Education and Research (PGIMER), India
Sherry Dingman, Marist College, United States

***Correspondence:**
Linda L. Benskin
LindaBenskin@utexas.edu

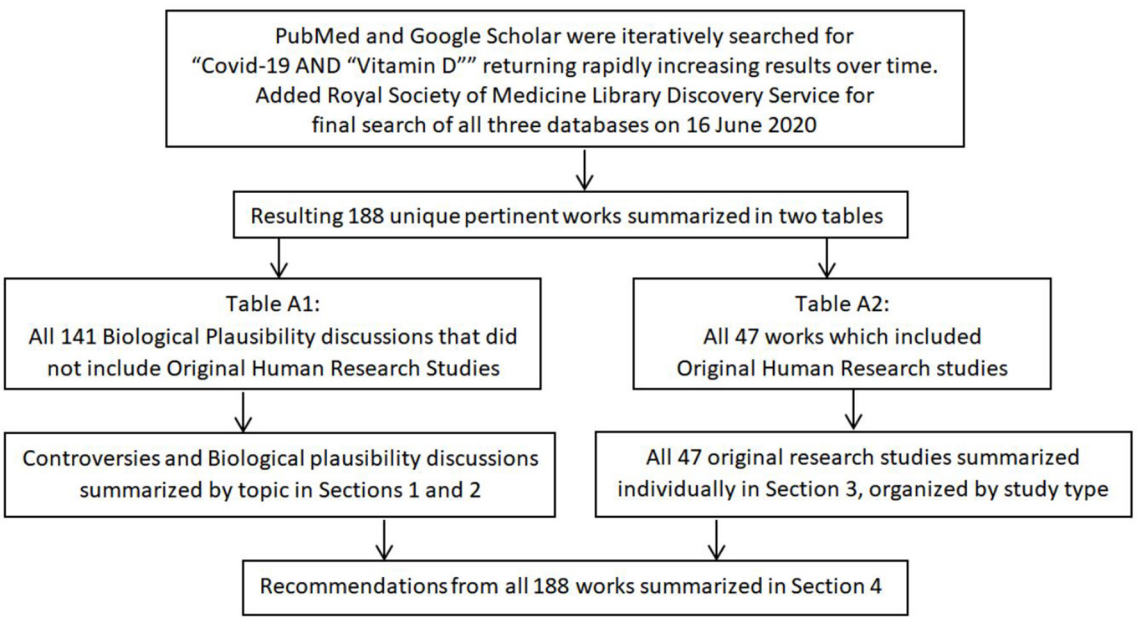
Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Public Health

Received: *13 May 2020*
Accepted: *07 August 2020*
Published: *10 September 2020*

Citation:
Benskin LL (2020) A Basic Review of the Preliminary Evidence That COVID-19 Risk and Severity Is Increased in Vitamin D Deficiency. Front. Public Health 8:513. doi: 10.3389/fpubh.2020.00513

rates (16 studies) and linearly predicted COVID-19 illness severity (8 studies). Two causal modeling studies and several analyses of variance strongly supported the hypothesis that vitamin D deficiency is a causal, rather than a bystander, factor in COVID-19 outcomes. Three of the four studies whose findings opposed the hypothesis relied upon disproven assumptions. The literature review also found that prophylactically correcting possible vitamin D deficiency during the COVID-19 pandemic is extremely safe. Widely recommending 2,000 IU of vitamin D daily for all populations with limited ability to manufacture vitamin D from the sun has virtually no potential for harm and is reasonably likely to save many lives.

Keywords: vitamin D, COVID-19, health disparities, minority health, vitamin D deficiency, preventive medicine



GRAPHICAL ABSTRACT | Search and reporting method.

INTRODUCTION

COVID-19 was first recognized in December of 2019 (1, 2[P])[1]. By January of 2020 it was clear the elderly are by far the most likely succumb to COVID-19 pneumonia, which is caused by a "cytokine storm." (6, 7). Later, male sex, obesity, and possessing naturally melanin-rich skin while living outside of the tropics came to be known as the highest risk factors after older age (2[P], 8–13, 14[P], 15, 16). Unlike influenza, children under age 10 are almost completely spared in COVID-19 (17, 18). This unusual risk factor pattern presented a mystery that spawned studies showing that COVID-19 fatalities are especially high in areas with lower levels of sunshine due to latitude or air pollution, except when population vitamin D intake is high (9, 10, 12, 19–24). In fact, the risk groups for severe COVID-19 match the risk groups for vitamin D deficiency exactly, and there is biological plausibility: vitamin D is known to modulate the immune system, helping prevent both an under-reaction that allows upper respiratory infections to be contracted, and the over-reaction referred to in COVID-19 as the "cytokine storm" (see section Biological Plausibility Discussions) (19, 25, 26). This review explores the evidence related to the hypothesis that vitamin D deficiency increases both COVID-19 rates and illness severity.

Abbreviations: RCT, Randomized Controlled Trial; 25(OH)D, serum vitamin D level; RAS, Renin-Angiotensin System; UV, ultra violet (light); UVI, ultra violet light index.

[1]Note: Because this is such a rapidly evolving topic, some of the references for this article were still in the preprint stage of publication when this review was finalized. In addition, three studies (3–5) from Southeast Asia, all with results consistent with that of other studies, may have relied upon data from unofficial sources.

The Vitamin D Debate

The discussion of idea that the top risk groups for severe COVID-19 complications tend to have vitamin D deficiency (**Table 1**) was initially popularized, not by the scientific community or

TABLE 1 | Classification of vitamin D levels (serum 25(OH)D levels): (3–5, 12, 27–39).

Classification	Nanograms	Nanomoles	Recommended D
Danger of toxicity	>100 ng/ml*	>250 nmol/l	
Normal or optimal	>30 ng/ml	>75 nmol/l	400–4,000 IU/day
Insufficient	21–29 ng/ml	51–74 nmol/l	4,000–6,000 IU/day
Deficient	11–20 ng/ml	26–50 nmol/l	7,000 IU/day
Severely deficient (often not distinguished from deficient)	<10 ng/ml	25 nmol/l	10,000 IU/day x 1 month or 500,000 IU x 1

**some sources found that 150 ng/ml was not harmful.*

governmental bodies, but rather, by some entertainers and bloggers, who recommended supplements to their audiences (40–42). This led some in the scientific community to respond with either agreement or disapproval. Trinity College Dublin researchers quickly issued a news release urging the Irish government to change their recommendations for vitamin D supplements in light of evidence of an association between vitamin D levels and COVID-19 mortality (43). However, most governments, medical organizations, and key opinion leaders give one or more of these four reasons not to recommend vitamin D supplements: past claims for vitamin D benefits were overstated, evidence for a link to COVID-19 is insufficient, overdoses are theoretically possible, and the public might believe that taking vitamin D supplements will make them "immune" to COVID-19 (44–53).

Although the International Association for Gerontology and Geriatrics (IAGG) Asia/Oceania Region COVID-19 Prevention Statement acknowledged that increasing vitamin D levels could reduce infection risks in elderly individuals whose levels are insufficient, they recommended only "getting enough sunlight in the morning" without mentioning supplements (54). Two May 2020 Centre for Evidence-Based Medicine rapid reviews concluded, without discussing any of the recent studies, that there is no evidence to support a role for vitamin D in prevention or treatment of COVID-19 or the cytokine storm (45, 55). Alarmed by the media response to a literature review suggesting a link between COVID-19 and vitamin D, two Brazilian medical associations jointly published a note stating that vitamin D supplements are only approved for bone health (56–58). The high mortality rates among minorities are providing momentum for various public health program expansions, which could diminish if vitamin D deficiency, rather than access to care and economic disparities, were found to be even a partial explanation (59–62). In addition, previous studies of dubious quality suggesting that vitamin D can "cure" various chronic illnesses and may be influencing the reluctance to recommend supplements for COVID-19 (63).

However, despite these concerns, former CDC Chief Dr. Tom Frieden recommended sunshine and up to 2,000 IU/day of vitamin D as a potential preventative for COVID-19, the British Dietetic Association recommended sunshine (or 400 IU/day for those are not able to go outside due to self-isolation), and former vitamin D skeptic Dr. JoAnn Manson's calls for daily vitamin D supplements (1,000–2,000 IU/day) during the COVID-19 pandemic—if vitamin D intake is low and going outdoors is not feasible—were published on both Medscape and WebMD (19, 64–67). Medscape published a second review of the topic by McCall, in which correcting possible vitamin D deficiency was characterized as "low hanging fruit" that has no downside (68). Mitchell's brief review (20 May 2020) in a Lancet-affiliated online journal also supported the vitamin D hypothesis (69). Authors of an early meta-analysis of nine studies found that a high percentage of COVID-19 patients are either vitamin D insufficient or deficient, and that countries with lower population vitamin D status have somewhat higher COVID-19 mortality rates and somewhat lower COVID-19 recovery rates (70). In Qatar, vitamin D for prevention of COVID-19 is being proactively delivered to the homes of high-risk diabetics (71).

Irish Medical Journal Debate on Vitamin D Supplements During the COVID-19 Pandemic

The Irish Medical Journal hosted a six-article formal debate on the topic in response to three published reports, including one by the researchers managing Ireland's part of the 26-country longitudinal study on aging (TILDA), in their May 2020 issue (72–75). All three reports strongly recommended vitamin D supplements to help protect all adults in Ireland from COVID-19 while they are "cocooning" (not going outdoors) (72, 73) (details in **Appendix**).

Debate Over Reports Using "Big Data" (the UK Biobank and EPIC, see Results and Retrospective Chart Reviews That Are Neutral or Strongly Oppose the Hypothesis)

Three research teams relied on the 2006-2010 UK Biobank data for the vitamin D levels included in their analyses of the relationship between COVID-19 incidence and vitamin D status (13, 76, 77). Roy et al., challenged the assertion that vitamin D levels are stable over time (important since levels were assessed 10–14 years prior to the pandemic) noting that the cited study only included women and followed up for only 5 years (13, 78, 79). In fact, the cited study (Meng et al.) found that, rather than being stable, the mean 25(OH)D increased significantly ($p < 0.05$) over the 5 years, and that the increase was driven by significant ($p < 0.05$) increases among participants who were initially at risk for deficiency; supplement intake and overall vitamin D intake increased significantly ($p < 0.05$) (78). Etsy criticized a UK Biobank study's assumption that, despite government's recommendations to supplement, the participants failed to correct any vitamin D deficiency revealed by their participation (13, 80). In their preprint, Darling et al., cited a different study to support their assertion that vitamin D levels are stable over time (76, 81). However, the Norwegians in the cited study had far higher vitamin D levels than the UK Biobank participants at their initial evaluation, a subset increased their 25(OH)D levels significantly ($p < 0.001$) by initiating supplements, and, as in the study by Meng et al., vitamin D levels did increase significantly for the group as a whole over time ($p < 0.01$) (81). The authors of the most recent article using the UK BioBank study data did not address the issue of the use of potentially no longer accurate 25(OH)D levels in their preprint, but added a reference to

the same Norwegian study as Darling et al., in their published article (77).

During the time frame of these three studies, COVID-19 testing in the UK was extremely limited (Raisi-Estrabragh et al., stated that most were tested only if hospitalized) (77, 79). Such limited testing, Roy asserted, raises the possibility that the authors of the first UK Biobank study accidentally included COVID-19 positive patients who were only moderately ill in their negative group (13, 79). It is also likely that the two UK Biobank studies authored later compared COVID-19 patients with patients who had serious illnesses such as influenza pneumonia, rather than with healthy individuals (76, 77). If vitamin D deficiency increases viral infection risk and severity as hypothesized, the patients in both arms of these studies could be expected to have higher deficiency and insufficiency rates than the general population. In fact, the research teams found high rates of vitamin D insufficiency and deficiency in both of their study groups (76, 77).

Three authors all pointed out that the UK Biobank studies failed to address the severity of the COVID-19 the patients experienced, which is critical to the question of whether or not vitamin D deficiency contributes to the potentially fatal cytokine storm (13, 76, 77, 79, 80, 82). Boucher cautioned against adjusting COVID-19 study data for obesity or dark-skinned ethnicity, providing empirical evidence that both directly lower 25(OH)D (83).

Grant and McDonnell formally responded to the first UK Biobank study, asserting that their multivariable model was over-adjusted because causal factors were treated as confounds, suggesting that the authors provide multiple analyses for transparency, including simple and complex models (13, 84). They also requested that the analysis be stratified by ethnicity, citing a previous study in which low 25(OH)D increased risk for preterm birth equally across ethnicities (84). They echoed Roy's concern that lack of a positive COVID-19 test result did not assure lack of infection in the UK at the time (84). Grant and McDonnell concluded by pointing out that few UK Biobank participants had 25(OH)D levels in the immune-protective range (>40 ng/ml), which would decrease the effect (13, 84).

In their response, rather than addressing the question of vitamin D deficiency being caused by old age, disability, obesity, etc., the authors of the first UK Biobank study stated that 25(OH)D cannot be a mediator because it is not the "cause" of old age, disability, etc. (84). They asserted that impaired health is more likely associated with reduced outdoor activity than with vitamin D status (84). They presented the non-significant results of an "intermediate" model that still included the deficiency-related variables of age, sex, ethnicity, and obesity, omitting only "health-related covariates" (e.g., BP, diabetes) as proof that inclusion of potential mediators did not influence their initial study results (84). The original study authors also reiterated their assertions that there is no statistical interaction between ethnicity and vitamin D deficiency and a positive COVID-19 test would ascertain more severe infections, and concluded by stating that 40 ng/ml is not deficient because in the adult UK population mean 25(OH)D levels are only 17.4 ng/ml for men under 65 years and 18.9 ng/ml for women under 65 years (84).

Although vitamin D levels are not drawn routinely, Fox used data from EPIC, a database with 15,000,000 patients across 26 states in the USA, in his analysis of the relationship between vitamin D status and COVID-19 infection, hospitalization, and mortality rates (85). DeFilipps commented succinctly, pointing out because vitamin D deficiency is ubiquitous, assuming patients with no 25(OH)D in their charts were vitamin D sufficient renders the study results study unreliable (86). DeFilipps recommended evaluating only the subgroup who were hospitalized for COVID-19 who had pre-existing conditions and known vitamin D deficiency to determine if there was a relationship between their level of deficiency, illness severity, complications, or length of stay (86).

Defining Appropriate Serum Vitamin D Levels and Appropriate Supplementation Dosages

The 25(OH)D (serum vitamin D level) is the most reliable indicator of functional vitamin D status, but until recently, the test assays varied (87). However, past research study results can be compared by mathematically harmonizing them, and increasingly, labs are adopting LC/MS ($D_{2+}D_3$) as the standard, increasing consistency (87).

Controversy Concerning Risk of Overdose

Food fortification was introduced shortly after the discovery of vitamin D. However, there was a dramatic increase in infants with hypercalcemia in the UK, leading to an abrupt scaling back of fortification (88). Later, a rare genetic defect, Williams–Beuren syndrome, was found to be responsible for the hypercalcemia (88). However, vitamin D toxicity concerns remain heightened, with a reluctance to recommend supplements (**Figure 1**). Dietary sources provide UK adults with only about 100 IUs of vitamin D per day (89, 90). During the COVID-19 pandemic, after concluding that vitamin D is likely to reduce acute respiratory tract infection risk, and that 10,000 IU/day is safe, the NHS paradoxically recommended only 400 IU/day "to protect bone and muscle health"(50, 90).

It is not considered possible to achieve toxic levels via the sun alone, and supplementation for prolonged periods brings 25(OH)D to toxic levels only if the dose is consistently extraordinarily high (40,000 IU/day for many months) (28, 88, 91, 92). The average naturally acquired 25(OH)D among equatorial tribal groups is 46 ng/ml (93). Healthy lifeguards typically have 25(OH)D levels of 100–125 ng/ml (29).

The Endocrine Society found toxicity symptoms only at levels above 150 ng/ml (93). Toxicity is related to high calcium levels; 25(OH)D levels higher than 150 ng/ml in conjunction with high calcium levels produce weakness, GI symptoms and accompanying weight loss, arrhythmias, confusion, and kidney damage (28, 88, 92). Historically, toxic levels of vitamin D (>150 ng/ml) have almost exclusively been the result of industrial errors (inaccurate doses in supplements or fortified foods), and the few cases of toxicity from extremely high doses being intentionally taken for prolonged periods of time (sometimes under the direction of a health care practitioner) were rarely severe (94, 95).

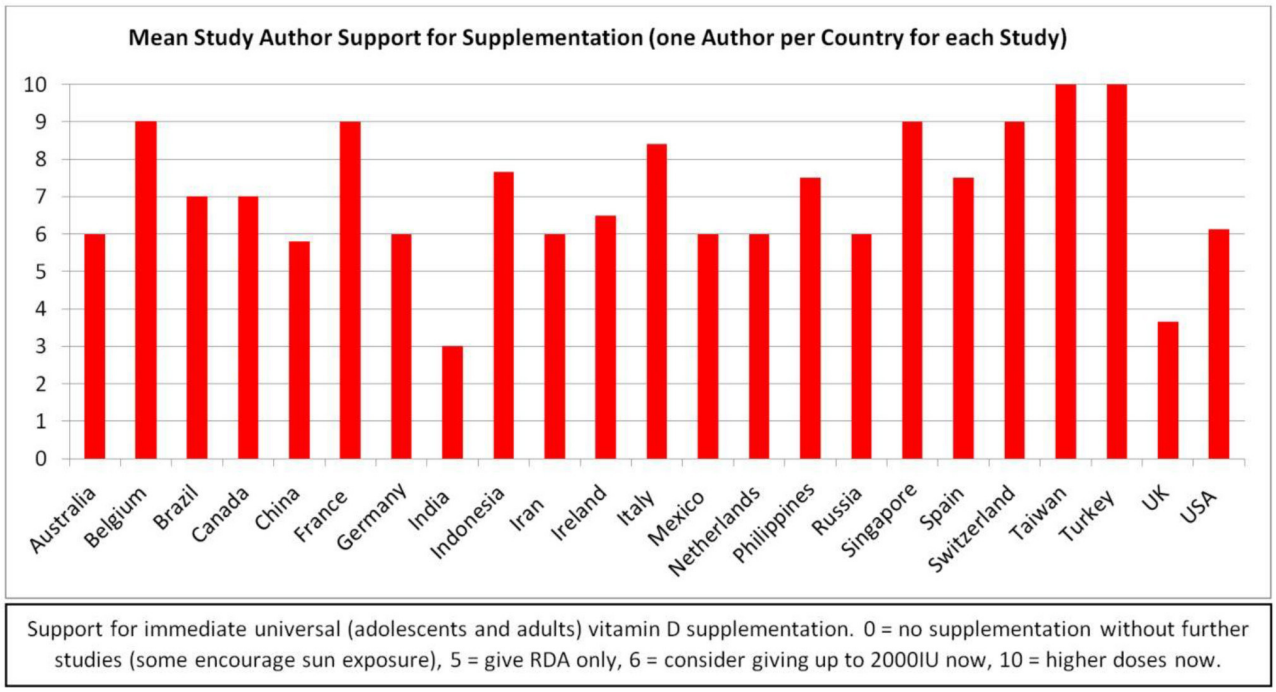


FIGURE 2 | Study authors' supplementation support by country.

Controversy Over Appropriate 25(OH)D Goals

In 2014, Veugelers and Ekwaru asserted that the statistical calculations to determine recommendations for vitamin D were incorrectly interpreted, leading to a US RDA (600 IU, or 700 IU/day for those over 70) that is off by a factor of more than 10 (87, 96). Heaney et al., supported the higher level in a reply, citing a recent supplementation study which supported an RDA closer to 7,000 IU/day (97). All three groups used the goal of 20 ng/ml for musculoskeletal health (70). In contrast, the Endocrine Society, aiming to optimize immune health and other aspects of vitamin D function, recommends adults take in 1,500–2,000 IU per day to maintain a 25(OH)D level of 30 ng/ml; 30 ng/ml is the NIH target level as well (29, 87).

Controversies regarding appropriate 25(OH)D, are also informed by studies of parathyroid hormone levels (29). Parathyroid hormone levels were not reduced in participants taking 15,000 IU/day, even with 25(OH)D levels above 60 ng/dl, in a study with a goal of bringing 25(OH)D levels up to at least 40 ng/dl (93). Mean serum calcium levels were not increased from baseline (93). 25(OH)D levels of up to 120 ng/dl appeared safe, and hypercalcemia and hypercalciuria were least common in participants with the highest 25(OH)D levels (calcium was not supplemented) (93). Goal 25(OH)D levels were achieved by 70% of the participants with 6,000 IU/day for normal weight participants, but 7,000 and 8,000 IU was required for overweight and obese participants, respectively (93).

Growing research suggests that 40–60 ng/ml is needed for prevention of respiratory infections, and 50–80 ng/ml is required to favorably influence hypertension and cardiovascular disease (28). In a 2019 randomized controlled trial, subjects without deficiency [initial 25(OH)D < 25 ng/ml] who took 10,000 IU/day for 3 years were slightly less likely to suffer a serious adverse event than those taking 400 IU/day (98). Mean 25(OH)D levels in the 400 IU/day group did not increase, while 25(OH)D for the 10,000 and 4,000 IU/day groups rose and then plateaued at 58 and 53 ng/dl, respectively (98).

Controversy Over Recommended Supplement Doses

Recommended upper limits of vitamin D supplements in the USA were relaxed after several studies demonstrated that 4,000 IU of vitamin D daily is safe (28, 75, 91). One review showed that 10,000 IU daily seemed to be the upper limit of tolerability (75). The Endocrine Society recommends up to 10,000 IU/day, particularly for obese individuals (93, 99). However, some study participants have taken 15,000 to 40,000 IU daily for at least 6 months without apparent adverse effects (91).

The European Society for Clinical Nutrition and Metabolism recommends a one-time dose of 500,000 IU IV for ICU patients who are vitamin D deficient (25(OH)D less than 20 ng/ml), based upon evidence that this practice decreases length of stay (12, 19, 100). Giving 500,000 IU enterally over 5 days increased 25(OH)D levels and decreased ICU length of stay, but giving the entire 500,000 IU in one bolus enterally did not improve 90-day mortality rates (101, 102).

Grant et al. authored an early article positing a relationship between COVID-19 and vitamin D which recommended 10,000 IU/day for 1 month, followed by 5,000 IU/day, with a goal 25(OH)D of 40–60 ng/ml (19). Although some other

researchers agreed, many were outraged (**Tables A1, A2**). Kow et al., questioned both the dose and the goal, citing a robust study in which supplements decreased the incidence of acute respiratory tract infections only when 25(OH)D levels were less than 10 ng/ml, and 800 IU/day was sufficient (103, 104). Grant et al., replied with several additional studies to support their recommendation of 40–60 ng/ml as a goal, but included an example of significantly decreased incidence of respiratory infections with lesser vitamin D_3 doses (although 800 IU was inferior to 2,000 IU, it still provided significant benefits over the placebo) (105, 106).

Sharma et al., reviewed the literature informing decisions about COVID-19 and vitamin D_3, finding compelling evidence for 10,000 IU/day for a month, followed by 5,000 IU/day to bring 25(OH)D levels up to the target of 40–60 ng/ml, then recommended a more modest 1000–2,000 IU/day (107). One group, Quesada-Gomez et al., posited that vitamin D supplementation should be with oral calcifediol (108). However, the majority of researchers and commenters recommend vitamin D_3 supplements of 1,000 IU−4,000 IU during "COVID-19 times," with a goal of achieving 25(OH)D levels of 30 ng/ml (see citations for **Table 1** and section COVID-19-Specific Recommendations of Experts) (109).

A meta-analysis of vitamin D supplementation to prevent acute respiratory infections found that daily vitamin D supplementation was safe and provided modest protective benefits, rising to a 70% protective effect when deficiency was corrected (104, 110). However, studies also found that large bolus doses are not particularly beneficial (104). Effective study doses of vitamin D were most often in the range of 400–2,000 IU (10–50 mcg), with the higher doses being given to adults (104). A 2020 study of pregnant women also found that daily supplementation is superior to boluses, that 2,000 IU/day was sufficient to resolve deficiency over time, and that up to 5,000 IU/day is safe (111).

METHODS: LITERATURE SEARCH

"COVID-19" is the MeSH term for SARS-CoV-2 disease, coronavirus 2019, COVID-19, and derivative terms. The topic of COVID-19 is a relatively new one, with the first reports published only 6 months ago (January 2020). In addition, because vitamin D supplementation is controversial, publication bias is a significant concern. Consequently, a significant percentage of the pertinent literature is found only on preprint services, most of which are captured by Google Scholar. PubMed casts a wider net than MEDLINE. Therefore, initially, PubMed and Google Scholar were searched for "COVID-19" AND "Vitamin D" (date range, 2020, omitting citations and patents, no language limitations). Repeated searches confirmed the growing interest in the hypothesis that vitamin D deficiency may play an important role in the COVID-19 pandemic (3, 10, 12, 19, 25, 110, 112–127) From May 2 to May 19, Google Scholar hits increased from 49 to 88 and PubMed hits increased from 17 publications to 32. By June 16, the Google Scholar search retrieved 158 possible references and the PubMed publications on the topic had increased to 69. Using the same search terms, the author also accessed the Royal Society of Medicine Library Discovery Service, which, on 16 June, 2020, provided 144 results from academic journals, reports, magazines, and electronic resources.

Duplicates were deleted and full texts obtained for every publication from all three sources as of June 16, 2020, references were scanned for additional sources, and appropriate articles found on ResearchGate and through other internet sources were added to capture how the topic is being addressed in the popular press. Authors of perspectives and studies on this topic span the globe (**Figures 2A,B**). Most of the research publications are quite brief, and many of the PubMed indexed articles are expert summaries of relevant data supporting the biological plausibility of the hypothesis, rather than reports of original research. Therefore, the author deemed it premature to limit this review to the "best evidence" as one would do in a formal systematic review of the literature. Rather, every publication discussing vitamin D with respect to COVID-19 found by the three formal searches as of June 16, 2020 is included in **Table A1** (Biological Plausibility and In Vitro Studies, $n = 141$) or **Table A2** (Original Research, $n = 47$). All original research studies (excepting *in vitro*) are summarized in the Results (section Results of Searches). However, due to space limitations, while many of the **Table A1** documents are cited, few are summarized individually.

RESULTS OF SEARCHES

Planned Clinical Trials

Formal clinical trials that include COVID-19 and vitamin D (including 16 on clinicaltrials.gov) include: vitamin D boluses plus other medications for COVID-19 positive patients, boluses, or moderate daily doses to prevent severe complications in at-risk populations, low-dose vitamin D as a placebo in a drug trial, genetic variant studies focused upon the interaction between vitamin D and COVID-19, and studies of vitamin D levels in patients with differing severities of COVID-19 illness (45, 128–145). As late as May 19, 2019, few studies had begun recruiting. Although most of these studies are not designed to determine if daily modest vitamin D supplementation decreases either the risk of contracting COVID-19 or its severity, Dr. Manson plans to test this hypothesis (66).

Testing the hypothesis that vitamin D deficiency prior to contracting the virus increases COVID-19 rates and severity necessitates screening participants for deficiency at enrollment. Failing to correct a deficiency being considered as a potentially significant risk factor for fatal COVID-19 complications would be unethical (146). Therefore, before and after population study designs (recommending supplements to groups known to be vitamin D deficient and observing if the groups' fatality rates decline) might be more feasible than randomized controlled trials (146).

Biological Plausibility Discussions

Many of the reports examining the relationship between vitamin D and COVID-19 present biological plausibility arguments. These reports are summarized in both **Table A1** and **Table A2**. The main arguments are presented concisely here, citing both COVID-19 specific and primary sources.

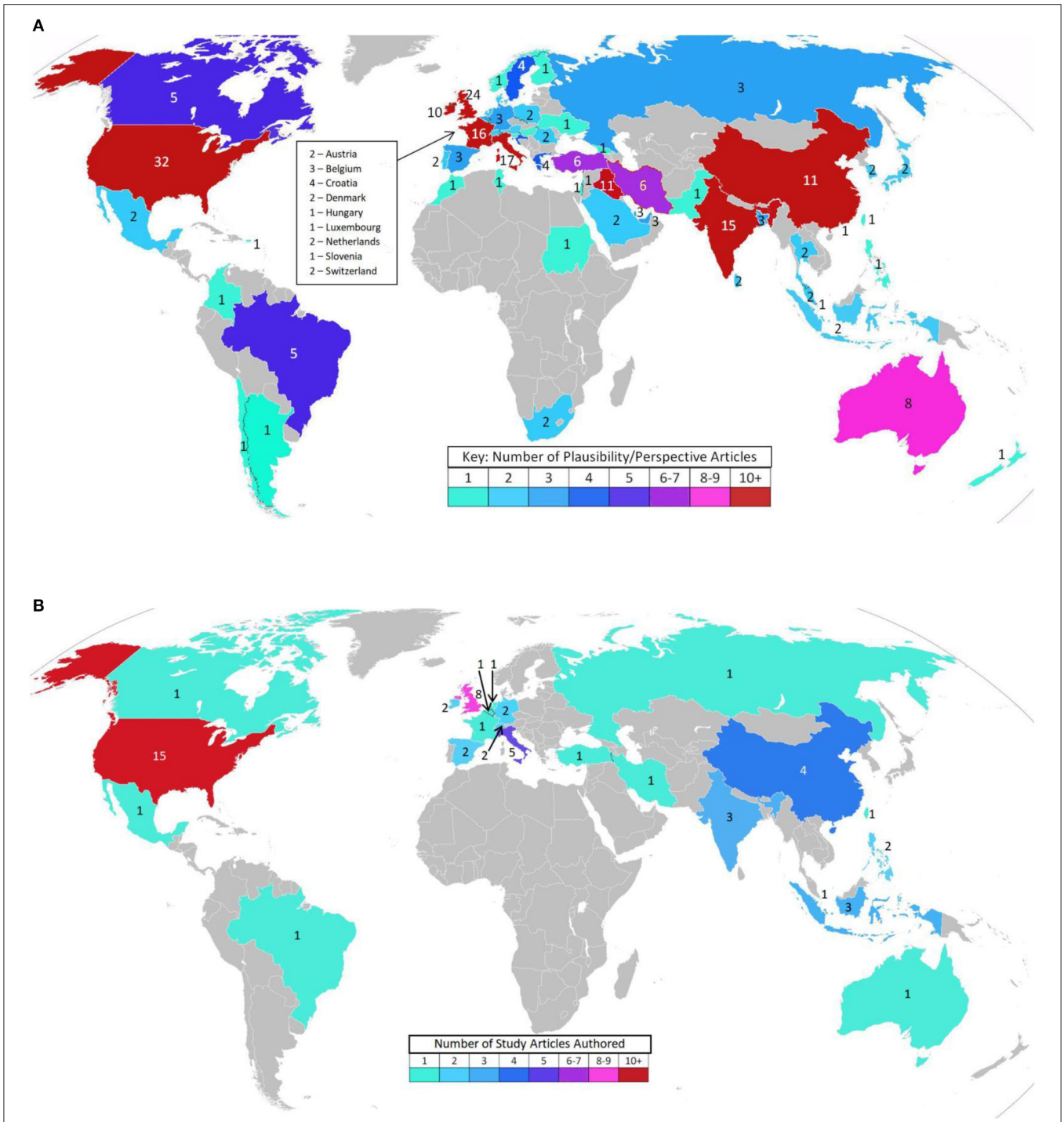


FIGURE 3 | (A) Interest in the topic—Biological plausibility article authors' locations (141 Articles). (Blank maps from wiki images: Creative Commons, credit: Canuckguy and many others). **(B)** Interest in the topic – Original research article authors' locations (47 Articles). (Blank map from wiki images: Creative Commons, credit: Canuckguy and many others).

Vitamin D Enhances Resistance to Viral Illnesses

Early studies of vitamin D supplementation for acute respiratory tract infections produced conflicting results (28, 104). Most studies of vitamin D for influenza prevention were conducted on healthy populations with high baseline levels, rather than on the deficient populations who would benefit most (19, 30, 121, 125, 147). Despite this, some found that higher 25(OH)D linearly enhance the innate immune response to acute winter respiratory

infections, halving the incidence and significantly reducing the duration of illness (31, 148, 149).

In 2017, 25 international researchers from 23 institutions performed a meta-analysis of individual participant data from 25 high-quality randomized controlled trials of vitamin D supplementation to prevent acute respiratory tract infections to determine why the results were inconsistent (104). They found that bolus doses were not consistently protective, even in severely vitamin D deficient populations (104). Removing bolus-dose data led to consistent findings of benefit, regardless of initial vitamin D status (104). Daily or weekly vitamin D supplementation was most beneficial for participants with baseline 25(OH)D <10 ng/ml (severe deficiency), providing more statistically significant ($p < 0.001$) protection than the ($p < 0.02$) protection vitamin D provided less deficient participants (104). The authors found that response to vitamin D supplementation is so variable that studies should base findings on changes in 25(OH)D levels, rather than relying upon the vitamin D dose given to each participant (30, 104). They also found that vitamin D supplementation is extremely safe: even large doses did not increase risk of serious adverse events, such as renal stones (104).

Historical data provides modest support for the hypothesis that populations with high vitamin D deficiency rates allow new pandemic viral strains to propagate more freely. The only recorded time period void of new strains of pandemic influenza is 1920–1957, and vitamin D food supplementation was most prevalent during the middle of that time period: from 1930 to 1950 (63). The COVID-19 pandemic began in Wuhan during a particularly dark January: 42% darker than their average January in 13 years (2007–2020) (150).

Several studies have shown that vitamin D decreases the severity of dengue fever (151). Oral vitamin D supplements of 4,000 IU for 10 days were significantly more effective than 1,000 IU in reducing dengue virus replication and controlling the damaging cytokine hyper-reaction (151–154). Vitamin D supplementation also reduced rotavirus replication in pigs (154). A recent review article by Sharma et al., summarized biological plausibility arguments and found that vitamin D deficiency is associated with a wide range of viral illnesses, and that vitamin D supplementation was both preventative and decreased severity, limiting hyper-inflammatory complications (107).

In the lungs, formation of the peptide LL37, an innate immune system component that, among other things, attacks enveloped viruses such as SARS-CoV-2 and modulates the immune system, requires sufficient vitamin D levels (28, 32, 117, 155). LL37 is inhibited by carbon and other nanoparticles in air pollution (32). Therefore, vitamin D deficient individuals can be expected to be at increased risk of both developing COVID-19 and experiencing the "cytokine storm" if they become infected, particularly in areas of the world with high levels of air pollution (32, 117).

How Vitamin D May Decrease Serious COVID-19-Associated Complications

During the "Spanish flu" pandemic of 1918-1919, deaths were substantially reduced when patients were treated in "open air" hospitals with access to sunlight, perhaps due to vitamin D's "cytokine storm" suppression (63, 150, 156). In the deep south, dramatically increased incidence of pneumonia led to much higher Spanish flu case fatality rates for African Americans than for whites (19). COVID-19 usually produces mild symptoms in the seemingly-vulnerable homeless, who are disproportionately outdoors, despite the population skew toward older males and African Americans (157–159). Prior to antibiotics, cod liver oil, UVB phototherapy, and sunshine, all of which are vitamin D sources, were considered successful treatments for tuberculosis (99).

Vitamin D enhances the innate immune response while, paradoxically, protecting against excessive inflammation by suppressing TNFα and the cytokines (e.g., IL-6, IL-17) implicated in severe COVID-19, and elevating anti-inflammatory IL-10 (19, 28, 31, 33, 45, 91, 149, 160–169). Many of the articles referenced here include detailed descriptions of the role of vitamin D in preventing a "cytokine storm" and several authors, including Meftahi et al., and Biesalski, added a series of cartoons to their papers to simplify the concept (167, 168).

Given that vitamin D decreases pro-inflammatory IL-6 and that IL-6 is implicated in the COVID-19 "cytokine storm," (170) and finding that mean IL-6 levels are higher in males and African Americans and increase with age and obesity (groups with increased risk for COVID-19 mortality), Silberstein went on to evaluate the possibility that vitamin D deficiency causes upregulation of IL-in high risk individuals prior to exposure to COVID-19, increasing their likelihood of developing fatal COVID-19 complications (171). Using detailed IL-6 data from Tuscany, Italy, Silberstein found a strong correlation between age stratified COVID-19 deaths in Italy and mean IL-6 levels [$r(6) = 0.9837$, $p = 0.00025$] (171). Data for a similarly detailed analysis for sex, obesity, and ethnicity was not available (171). The authors note that IL-6 is generally low in children, but it is high for a brief time in early childhood, which could explain the Kawasaki-like COVID-19 sequela in some children (171).

Vitamin D also helps prevent viral infections from progressing to pneumonia by tightening cell junctions (19, 28, 165, 172) And, vitamin D's influence on the coagulation pathway decreases risk of acute respiratory distress syndrome as it decreases thrombosis risks (114, 127, 157, 162, 169, 173). Therefore, correcting vitamin D deficiency might help prevent COVID-19 illness AND help limit complications when prevention is unsuccessful (25, 28, 114, 169).

Daneshkhah et al., proposed that Vitamin D deficiency causes C-reaction protein (CRP) levels to rise, thus increasing the likelihood of a cytokine storm (174). The authors found that CRP and vitamin D status are inversely related in healthy individuals (174). CRP levels were increased in severe COVID-19 patients, but because CRP is a marker for inflammation, it was unclear if this was a cause or an effect (174). The authors used population data from 10 countries to show a possible link between vitamin D status and the adaptive average case mortality ratio, and provided significant biological plausibility arguments in support of the hypothesis (174). The authors proposed further studies to determine if COVID-19 patients with high CRP are deficient in vitamin D (174).

Risk for Severe COVID-19 Parallels Risk for Vitamin D Deficiency

Many authors, some with compelling statistical analyses, propose vitamin D deficiency from low sunlight levels (Nordic countries have high vitamin D intake) to explain the geographic distribution of severe COVID-19 (9, 10, 26, 34, 112, 122, 150). Italy and Spain have very high vitamin D deficiency rates (12, 27, 34, 63, 122, 175). First-generation non-Western immigrants, even in countries with low overall rates, are often vitamin D deficient (176–178). Vitamin D deficiency is especially common in the elderly, in part because synthesis from sunlight is muted in old age (19, 31, 34, 116, 122, 179–182). Naturally melanin-rich skin increases vitamin D deficiency risks, particularly in high latitudes (13, 34, 112, 116, 122, 148, 181). It takes significantly more sunlight exposure for someone with dark skin to attain the benefits that someone with lighter skin receives (14). Lower 25(OH)D is associated with diabetes, hypertension, cardiovascular disease, and COPD risk (19, 25, 31, 34, 183). Dialysis patients are often severely vitamin D deficient (184). Up to 50% of US nursing home patients, and 75% of institutionalized people in general, are vitamin D deficient (122, 182, 185).

The UK's low sunlight levels have been posited in the public press as an explanation for why health workers with naturally melanin-rich skin (mostly nurses and physicians) are so disproportionately represented on the Telegraph's tribute wall (186, 187). The only postpartum COVID-19 fatality in the UK was a vitamin D deficient diabetic Pakistani woman who suffered a thrombotic complication (188).

Current increased "stay at home" regulations and increased boredom and stress can be expected to result in eating patterns which increase obesity and the comorbidities with which it is associated (189). In part because vitamin D is fat-soluble, obese individuals have increased daily vitamin D intake requirements and are often deficient (25, 34, 57, 91, 175, 190, 191). In addition, vitamin D deficiency causes the body to store more fat by increasing parathyroid hormone levels (192). Obesity is a major risk factor for fatal COVID-19 complications, particularly in younger adults (34, 190). Ekiz et al., found that increasing vitamin D levels makes it easier to lose excess weight, which could lower individual COVID-19 risk (192).

Recent studies in Ireland and Switzerland both found that older males are at even higher risk of vitamin D deficiency than older females (72, 124). Vitamin D deficiency increases the X-chromosome linked "Renin-Angiotensin" System (RAS) activity, making men more susceptible to ACE2 receptor dysregulation and theoretically, to increased COVID-19 morbidity (25, 33, 123, 165, 193, 194). Although vitamin D deficiency is not universal in severe COVID-19, every deleterious symptom can be explained by RAS over-reaction, which would occur more easily in individuals without sufficient vitamin D to control the RAS (25, 126, 165, 183).

Evidence Informing the Hypotheses That Vitamin D Deficiency Influences COVID-19

While data from randomized controlled trials is superior, the hypothesis that vitamin D deficiency is a major contributor in COVID-19 risk and severity is already supported by 20 population-data analyses, both causal inference modeling reports, four case studies/series, one prospective correlational study, one case control study, one cohort observational study, and 10 retrospective chart reviews. One population-data analysis and three retrospective chart reviews supported the dissenting view. One population-data analysis, one retrospective chart review, and the lone systematic review were neutral. Recognizing that truth is not exposed by the mere tallying of positions, but rather, by evaluating the specifics of the data and the strength of the study designs, all 47 studies are summarized here and in **Table A2**.

Analyses of Population Data

Bäcker asked whether temperature or radiance could explain the speed and level of geographic spread of COVID-19 (150). Every location with over 2000 cases by March 15, 2020 had an average temperature of 10°C or lower (150). And, over a longer time period, locations with 4-week temperature averages under 14°C when they reached 100 cases all had faster growth than any of the warmer locations ($p = 0.0001$) (150). The same analysis using deaths instead of cases yielded a similar negative correlation ($p < 0.02$) (150). However, Finland, Norway, and Russia, all reaching 2000 cases after March 15, did not conform to the pattern, leading to a study of sunlight (150). Indeed, irradiance and cloudopacity better accounted for all the of data ($p < 0.01$) (150). Bäcker suggests (with data to back up his hypothesis) that increased cloudiness and air pollution can explain why in Korea, Daegu had 10 times as many cases of COVID-19 as more internationally-connected Seoul, and in Italy, Lombardy had over 10 times as many cases as more internationally-connected Lazio (Rome) (150). Multivariate regression found that the best independent predictor of COVID-19 case ($p < 0.001$) and death ($p < 0.001$) growth rates was the average zenith (most direct sun rays) when the location reached its 100th case (150) Zenith, correlated with both irradiation ($p < 0.01$) and temperature ($p < 0.001$), explained the lower growth rate in Finland, Norway, and Russia, and fully accounted for the variance from both (150). No association with increased travel or visiting was found (150). Bäcker concluded that sunlight leads to less COVID-19 transmission, likely due to both the direct result of irradiation and increased vitamin D, and thus, advising people to stay indoors rather than opening up outdoor recreation areas during the COVID-19 pandemic appears to be a poor choice (150).

In contrast, Yao et al., found no association between COVID-19 transmission rates and temperature or UV radiation across the 62 cities (of 224) in China with at least 50 cases at the peak of the outbreak (10 Feb) and at least 10 cases remaining on 9 March (195). However, the authors note that their study examined data from early January to early March, 2020, a time during which strict travel restrictions were put into place to prevent COVID-19 transmission in China (195, 196). It is possible that many of the cities in which UV light or temperature effectively reduced transmission were eliminated from the study because they no longer had the minimum of 10 cases by 9 March.

Two statistical analyses of geographical areas in the USA addressed the question of whether high COVID-19 fatality rates

in African Americans could be explained by income levels (14, 197). Bäcker's initial statistical analysis from 8 cities and states that provide a racial breakdown of COVID-19 victims found that race-based fatality rate differences diminish in direct proportion to available sunlight, with COVID-19 deaths among blacks in Detroit at 193% higher than the percent-black area population, but only 7% higher in Florida (Pearson −0.76, $p < 0.05$) (14). African Americans comprise 26% of Milwaukee County's population, half of their COVID-19 cases, and 81% of its deaths (14). This led to an exploration of the hypothesis that rather than socioeconomics (lower incomes, jobs that do not permit social distancing) being solely responsible, irradiance may play a large role in the disproportionate COVID-19 morbidity and mortality rates among African Americans in the USA (14). In Michigan, the state with the highest racial disparity in COVID-19 deaths, a county-by-county analysis showed that percent African American, but not percent over 65 years, median income, median age, or number of people per household, significantly ($p < 0.05$) correlated with COVID-19 morbidity (14).

Similarly, Li et al., focused on US counties with at least 50 COVID-19 cases (661 counties) and those with at least 10 deaths (221 counties), grouping them into quartiles and comparing highest to lowest (197). Multivariate analysis demonstrated that "percent black" predicted county cases and fatalities, even after controlling for other demographics, socioeconomics, and comorbidities (197). Higher daily temperatures decreased county case numbers, but not mortality rate (197). They proposed vitamin D deficiency among black Americans as a "unifying theory" to explain their results (197).

Laird et al., plotted COVID-19 mortality/million against mean 25(OH)D levels for twelve European countries, finding a significant correlation ($p = 0.046$) (27). Panarese and Shahini ranked the 108 countries with at least 100 COVID-19 cases on 2 April 2020 by latitude, demonstrating visually that, overall, deaths per million were higher in the northern-most countries, whose citizens would be the most likely to be vitamin D deficient from the dark winter (198). Following up on Panarese and Shahini's work, Rhodes et al., compared the 120 countries with more than 150 COVID-19 cases by 15 April 2020, finding that COVID-19 mortality rates were significantly correlated with latitude ($r = 0.53$, $p < 0.0001$) (26). Rhodes et al., used a simple scatter-graph to illustrate that the COVID-19 mortality rates per million population were dramatically lower in countries with capitals south of 35°N, where sunshine in the time immediately preceding the pandemic made maintaining vitamin D levels possible (26).

Ilie et al., reported a significant correlation between low mean vitamin D levels across 20 European countries and both COVID-19 fatalities/million population ($p = 0.05$) and COVID-19 cases/million population ($p = 0.050$) (122). Kumar and Srivastava objected to Ilie et al.,'s study, stating that the correlation was being stretched by the media to claim that vitamin D supplements may reduce COVID-19 mortality rates by 50% (199). Expressing concern that this exaggerated claim would lead to fatal overdoses, the authors conducted a statistical analysis of COVID-19 case and death rates and life expectancy using the data from Ilie et al. (122, 199). The authors asserted that because vitamin D deficiency increases with age, controlling for life expectancy would reveal the true relationship between vitamin D and COVID-19 infection and fatality rates (199). The researchers found that life expectancy was a better predictor of both COVID-19 mortality and case rates than vitamin D (199). Kumar and Srivastava did, however, call for clinical trials of vitamin D supplementation (199).

Citing Ilie et al., Singh et al. compared mean 25(OH)D levels and COVID-19 cases and deaths per million population for 20 European countries on 8 April and again on 12 May (200). The significance of the inverse correlation between vitamin D and case rates increased from $r_{(20)}$: −0.4435; $R^2 = 0.1967$ ($p = 0.0501$) in April to $r_{(20)}$: −0.5504; $R^2 = 0.3029$ ($p = 0.0119$) in May (192[P]). However, the inverse correlation for death rates decreased from $r_{(20)}$: −0.4378; $R^2 = 0.1917$ ($p = 0.0535$) to $r_{(20)}$: −0.3935; $R^2 = 0.1549$ ($p = 0.0860$) (200). Singh et al., did not discuss the possibility that vitamin D levels increased between April and May as sunshine increased, potentially protecting patients from the "cytokine storm" (200).

Notari and Torrieri's much larger, more detailed, comprehensive 126 country data review found that most of the 24 identified potential risk factors for COVID-19 propagation, including blood type, life expectancy, and even greeting habits, were significantly correlated with one another (201). Prevalence of Type-I diabetes, BCG vaccination, and vitamin D levels were the only "almost independent factors" (201). In the 42-country subset with vitamin D data and high GDP, lower mean annual levels of vitamin D were linearly related to increased COVID-19 risk ($p = 0.006$), with seasonal values (March) demonstrating even more significance ($p = 0.002$) (201).

Kara et al., mapped the population prevalence of vitamin D deficiency (<20 ng/ml) and severe deficiency (<10 ng/ml) against COVID-19 total fatalities for the 40 most affected countries, worldwide, finding a clear relationship (34). Regression analyses demonstrated a quadratic relationship between prevalence of vitamin D deficiency and insufficiency and COVID-19 cases (34). A histogram with regression lines illustrated the relationship between latitude, population vitamin D status, and country rank (by number of cases) (34). Finding vitamin D deficiency and COVID-19 to be related pandemics, they agreed with Grant et al., in recommending vitamin D (without high calcium) supplementation, as well as encouraging fortified food intake and increased sun (UVB) exposure (34).

Braiman noted that as of March 22, 2020, although 10% of the COVID-19 cases lived south of the Tropic of Cancer, they represented only 1% of the fatalities (146). The three exceptions could all be explained by mean population vitamin D levels (146). Nordic countries have vitamin D deficiency rates below 1% (due to diet or supplementation) and impressively low COVID-19 fatality rates, except Sweden (146, 202). In Stockholm, severe vitamin D deficiency is common among displaced Somalis, who with less than 1% of the population have suffered 40% of the COVID-19 fatalities (10, 178, 203). Indonesia straddles the equator, but its predominately Muslim women have vitamin D levels that are only half that of notoriously low Italy (146). Sunscreen use is popular in the Philippines, which may account for the high levels of vitamin D deficiency there (146). Braiman recommended ethical testing of the hypothesis that

vitamin D and COVID-19 outcomes are related by encouraging supplementation in deficient populations and evaluating death rate changes (146).

Although Latinos and African Americans were found to be at higher risk of COVID-19 mortality in New York City, it is difficult to determine the influence of Latino ethnicity vs. race (over 75% of Latinos identify as non-white), because New York City does not provide sufficiently detailed data (204). In contrast, Georgia does break down COVID-19 data by both ethnicity and race (204). Black Latino COVID-19 morbidity was 123% higher than white Latino morbidity ($p < 0.001$), supporting researcher Bäcker's hypothesis that a darker complexion decreases sun exposure benefits (204). COVID-19 morbidity is 37% higher for white non-Latinos than for white Latinos ($p < 0.0001$), 689% higher for Native American non-Latinos than for their Latino counterparts ($p < 0.01$), and there were no cases of COVID-19 among Latino Asians (204). Latinos spend more time outdoors than any other racial group (85% more than African Americans) which could explain why Latinos defied externally-imposed racial disparity explanations (204). The author concluded that irradiance exposure seems to help prevent COVID-19 (204).

Countries with higher rates of vitamin D-rich sea fish consumption or food supplementation have lower COVID-19 mortality rates than adjacent countries (182). The elderly, especially in nursing homes, where 84–93% of residents in the US are vitamin D insufficient, are at highest risk for severe COVID-19 (182). Bäcker and Mageswaran evaluated vitamin D deficiency rates among elderly females and COVID-19 deaths prior to May 31st in 32 countries, finding that case fatality rates were up to twice as high in countries with high vitamin D deficiency rates ($p < 0.04$) (182). They also found that case fatality rates were significantly higher ($p < 0.026$) in countries with a high percentage of black inhabitants (182). Noting many biological plausibility arguments and vitamin D deficiency and insufficiency race disparities, the authors recommend COVID-19 prevention and treatment studies (182).

Li et al., used machine learning to produce logistic models to predict case rates, death rates, and case fatality rates in all 50 US states and 154 countries listed on the Johns Hopkins COVID-19 dashboard on 15 May 2020, assessing the interdependence of the 57 factors LASSO identified as potentially influencing COVID-19 outcomes (205). Among their many findings, Li et al., determined that higher population vitamin D intake is an independent factor in reduced COVID-19 cases (205).

Kohlmeier performed a Mendelian randomization to test the effect of latitude (a proxy for vitamin D) on rates of African American COVID-19 deaths in the 22 reporting states with more than 15 African American deaths as of 16 April 2020, finding a strong relationship ($r = 0.427$) (206). A correlational analysis found that excess mortality rates were significantly higher ($r = 0.435$, $p = 0.02$) in states with higher latitudes (206). The highest excess mortality rates were all in states near or above 40° N, where UVB intensity in winter and spring is too low to provide vitamin D (206). The African American fatality over-representation was 5.6-fold in Wisconsin compared with 1.3-fold in Florida (206).

Adding the proposed relationship between latitude and COVID-19 to knowledge that ozone filters the ultraviolet-B radiation the body requires to produce vitamin D, Alipio evaluated data from all 34 countries with April 2019 ozone data available on an open-access database (207). Kendall rank correlation test found that ozone concentration significantly ($P < 0.001$) positively correlated with COVID-19 cases, but latitude and COVID-19 cases appeared to have no relationship (207).

Recognizing the advantages of comparing cities within a single country with varied UV radiation, altitude, and weather patterns, such as consistent policies, culture, and genetic factors, Skutsch et al., conducted a multiple regression analysis of data from 45 cities in Mexico, comparing the rate of increase in cumulative COVID-19 cases and fatalities (208). Data from January was included because, while UV light's sterilization effect would be immediate, physiologic vitamin D formation precedes its impact on infection and mortality rates (208). Skutsch et al., found a negative relationship between rate of transmission and altitude ($r = -0.354$, $p = 0.014$), but temperature, relative humidity, and latitude were insignificant (208). UV levels in January correlated a bit more strongly with transmission rates ($r = -0.369$, $p = 0.014$) than UV levels during the transmission period ($r = -0.32$, $p = 0.032$), supporting the hypothesis that the influence of UV is due to vitamin D rather than sterilization (208). In contrast, UV was only marginally associated with rates of mortalities (208). Mexico City's air pollution may have explained this (208). Surprisingly, altitude and UV levels were not significantly interrelated, but their combined effect accounted for 18% of transmission rate variation ($p = 0.0062$) (208). Data for 834 individuals scattered across 561 municipalities showed that lower altitude is a highly significant ($r = -0.35$, $p = 0.0005$) predictor of vitamin D levels, perhaps influenced by the high levels of UV light in coastal cities and the cooler climate of higher altitude cities leading to more clothing coverage (208).

Noting that all five US states with fatalities greater than 5,000 and four of the five states with cases over 90,000 are in latitudes above 37°N, Li, et al., used latitude as an indicator to evaluate the relationship between sunlight, vitamin D, and COVID-19 case and death rates per 100,000 population (209). Aggregate data (22 Jan−23 May 2020) showed that states in latitudes above 37°N, when compared with states at lower latitudes, had significantly higher case rates (702 vs. 255/100 K) and death rates (43 vs. 11 deaths/100 K) ($p < 0.001$) (209). The higher case rates were not attributable to higher test rates (209). The authors suggested sunlight and vitamin D as the explanation, calling for studies to evaluate the impact of vitamin D on the prevention of COVID-19 (209).

In a less detailed study, Marik et al., evaluated the case fatality rates for all 50 US States, mapping the results to illustrate that, with the exception of states with very low population densities and Louisiana, case fatality rates increased with increasing latitude (210). The cumulative summary case fatality rate for states over 40°N was significantly higher than for states below 40°N (6.0 vs. 3.5%, $p < 0.001$) (210). Attributing the differences to vitamin D's dampening of excessive inflammation, they advocated for standard vitamin D supplement doses and further studies (210).

Moozhipurath et al., obtained UVB radiation data for 108 days (through 8 May 2020) in the 152 countries with more than 20 COVID-19 cases, beginning when the country had over 20 cases, analyzing the relationship between daily UV index (UVI—a surrogate for UVB), COVID-19 deaths, and COVID-19 cases, controlling for weather variables, including ozone levels (24). UVI increase was associated with a 1.2% decrease in the daily growth rate of cumulative COVID-19 deaths ($p < 0.01$) and a 1.0% decrease in the daily growth rate of cumulative COVID-19 case fatality rates ($p < 0.05$) (24). The authors asserted that their methods led to very conservative estimates of the effect of UVB on COVID-19 deaths, and advocated for "sensible" increased exposure to sunlight, particularly for people at high risk of vitamin D deficiency (24).

A statistical analysis by Davies et al., found that COVID-19 outbreaks with large fatality rates occurred exclusively above 30°N, with a 55:1 ratio between 30 and 55°N and more southern latitudes (63). The Epidemic Severity Index was greater than 2.5 in nine of 239 locations, all above 30°N (63). Northern outliers all had higher vitamin D population levels, southern countries with the most severe outbreaks (Philippines and Brazil) have a high vitamin D deficiency prevalence, and fatality rates are doubled by naturally melanin-rich skin in the USA and UK (63). Iran, where religious full-body clothing is worn and vitamin D deficiency is common, fared far worse than Israel, whose vitamin D deficiency prevalence is relatively low (63).

Causal Inference Modeling Reports

Davies et al., also analyzed three potential root causes for their influence on COVID-19 outcomes, categorizing factors as lowering vitamin D, negatively influenced by low vitamin D, or vitamin D neutral (63). Environmental conditions hostile to the virus and environmental measures (e.g., distancing) decrease COVID-19 spread, but do not influence case fatality rates (63). If vitamin D is a "bystander" variable (simply a marker of bad health), case fatality rates would correlate best with vitamin D-neutral comorbidities (63). The authors provide a detailed analysis of the known COVID-19 epidemiological, latitude, and environmental data (63). A table illustrates that 16 predictions of the causal model accurately match the known facts, while 14 predictions of the bystander model strongly contradict the data and two more are not supported (**Figure 3**) (63).

Annweiler et al., used Hill's methodology for determining causality, which states that the more of the seven criteria are met, the stronger the claim, to evaluate the hypothesis that vitamin D is causally linked to COVID-19 outcomes (211). Vitamin D met six of the criteria, failing only on specificity (because vitamin D deficiency is high in the general population) (211). Concluding that vitamin D deficiency is highly likely to be a cause of poor COVID-19 outcomes, the authors suggest that these results, coupled with the excellent safety profile of vitamin D and lack of other treatments, support testing vitamin D as an adjuvant treatment and prophylaxis for the general population (211).

Case Studies and Case Series in Which Vitamin D Is Mentioned

Ahmed et al., reported that a COVID-19 positive maternity patient with diabetic ketoacidosis, vitamin D deficiency, and a history of asthma developed a fatal thrombosis 4 days post extubation (188). Horowitz et al., reported on two COVID-19 pneumonia patients with histories of immunosuppression from Lyme disease who responded to repeated doses of glutathione, along with a multitude of other drugs and remedies (212). One had a history of low vitamin D (212). Bossoni et al., reported on a 72-year-old thyroidectomized COVID-19 positive patient who experienced sudden onset severe hypocalcemia (213). Her parathyroid level was low, and she was extremely vitamin D deficient (8 ng/ml) (213). Bossoni et al., noted that home confinement can worsen vitamin D deficiency, increasing the risk of systemic infections and potentially life-threatening hypocalcemia (213).

Vitamin D deficiency is common in Indonesia, affecting 35.1% of elderly institutionalized women and 23% of the general population (214). Pinzon et al., tested 10 PCR-positive COVID-19 patients in Indonesia, finding that nine were vitamin D deficient (25(OH)D <10 ng/ml) and the remaining patient was

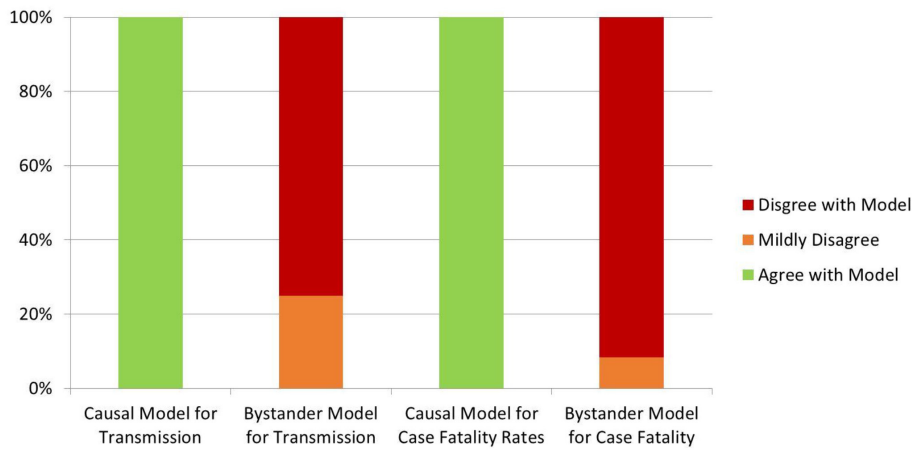


FIGURE 4 | Davies et al., Causal model results (63).

insufficient (25(OH)D = 20.5) (214). Finding no clinical evidence to inform the decision to provide vitamin D supplements to prevent or treat COVID-19 in their review of the literature, they called for randomized controlled trials and now prescribe all patients 2,000 IU/day (214).

Prospective Correlational Study, Case-Controlled Survey, and Cohort Observational Study

Vitamin D deficiency is common among Irish males (median 25(OH)D of 18.8 ng/ml for ages 40–60) (215). Faul et al., drew 25(OH)D in 33 COVID-19 positive Caucasian males over the age of 40 who were admitted to the hospital in respiratory failure without cancer, diabetes, cardiovascular disease, or chronic immunosuppressant intake in Ireland in March of 2020 (215). The 12 requiring mechanical ventilation (including all four fatalities) had mean serum 25(OH)D levels of 10.8 ng/ml, compared with 16.4 ng/ml for those requiring only oxygen ($p = 0.03$) (215). Patients with 25(OH)D <12 ng/ml had a hazard ratio for requiring ventilator care of 3.19 ($p = 0.03$) (215). The authors concluded that low vitamin D is either a marker for poor health, or it permits pro-inflammatory changes that lead to severe COVID-19: "a thought worthy of further study" (215).

Concerned about the effects of COVID-19 on their community-dwelling Parkinson's Disease patients in Lombardy, Italy, Fasano et al., conducted telephone interviews with 1,486 patients and 1,207 family-member case controls (216). The 105 Parkinson's patients with COVID-19 and 92 family members with COVID-19 differed only in decreased shortness of breath ($p = 0.004$) and decreased hospitalization rates ($p = 0.018$) for the Parkinson's patients (216). The authors adjusted the data for the age differences between groups, thought to be due to aggressive protective measures for the elderly in the area (216). Parkinson's, hypertension, and COPD medications did not influence the likelihood of developing COVID-19, while Vitamin D supplementation was protective ($p = 0.048$).

Tan et al., compared the 26 consecutive patients 50 years or older not requiring oxygen on admission who were hospitalized immediately prior to initiation of a daily oral combination of 1,000 IU vitamin D_3, 150 mg magnesium, and 500 mcg vitamin B_{12} with the next 17 consecutive patients meeting the same criteria to determine if these supplements altered support needs (217). Of the 9 patients supplemented within a week of symptom onset, only one required oxygen, and that was within 24 h of supplement initiation (217). Of the 8 patients supplemented over a week after symptom onset, one required ICU care within 24 h of supplement initiation, and one required oxygen 3 days later (217), Supplemented patients were less likely to need any oxygen (17.6 vs. 61.5%, $p = 0.006$) or ICU care (5.9 vs. 30.8%) (217).

Retrospective Chart Reviews Favoring the Hypothesis

Alipio performed a chart review using de-identified data from 212 COVID-19 patients with recorded pre-COVID-19 25(OH)D levels from three hospitals in Southern Asia in which 25(OH)D was tested initially and weekly (3). Individuals' 25(OH)D levels did not vary significantly during hospitalization, confirming that battling COVID-19 does not, in and of itself, deplete vitamin D (3). Vitamin D status (3 categories: >30, 21–29, or <20 ng/ml) correlated significantly and linearly with more critical COVID-19 illness (4 levels clearly defined by previous researchers) (3). For each standard deviation increase in serum 25(OH)D, the odds of having a mild, rather than a critical, case of COVID-19 were almost 20 times as great (OR = 0.051, $p < 0.001$) (3) (**Figure 4**).

A statistical analysis by D'Avolio et al., of records in a Swiss clinic's database for 107 symptomatic individuals obtaining a SARS-CoV-2 PCR test found that the 27 PCR-positive patients had significantly lower ($p = 0.004$) 25(OH)D (11.1 ng/ml) when compared with test-negative subjects (24.6 ng/ml) (124). PCR-positive patients were 70.4% male, while PCR-negative patients were only 48.8% male, with similar ages (124). Differences between 2019 median 25(OH)D verses PCR-positive 2020 median 25(OH)D were significant for both women (25.6 vs. 9.3 ng/ml, $p = 0.019$) and men (22.9 vs. 11.4 ng/ml, $p = 0.005$), but not for PCR-negative for either gender (**Figure 5**) (124).

Lau et al., found that among all 20 COVID-19 patients with recorded 25(OH)D at a New Orleans hospital, every ICU patient under age 75 had vitamin D insufficiency (157). Eleven of 13 ICU patients had vitamin D insufficiency verses 4 of 7 patients with milder COVID-19 (157). Seven ICU patients had critically low 25(OH)D (<20 ng/ml) and three had levels below (10 ng/ml) (157). Patients with the lowest 25(OH)D levels were African American (**Figure 6**) (157).

In Jakarta, Indonesia, hospitals are designed to provide patients sunlight and home patients exercise outdoors (218). In this setting, daily minutes of sunshine were compared with patient recovery, death rates, and incidence (218). Asary and Veruswati found sunshine was not related to prevention, but COVID-19 patient recovery briskness was significantly (Spearmen's $\alpha = 0.05$) correlated with sunnier days (218).

Sun et al., conducted a 241 patient retrospective chart review in a hospital in Wuhan, China, using standardized definitions of mild, moderate, severe, and critical COVID-19 (219). On admission, 74.7% of patients were hypocalcemic (219). Noting that vitamin D deficiency can cause hypocalcemia, the researchers found a median 25(OH)D of 10.20 ng/ml (severe deficiency) among the 26 patients tested; none were vitamin D sufficient (219). These 26 patients had worse CRP ($p < 0.001$), D-dimer ($p < 0.001$), and parathyroid hormone ($p = 0.048$) levels (219). Calcium levels positively correlated with 25(OH)D levels ($p = 0.004$), and lower calcium levels correlated linearly with lower SpO_2 levels ($p < 0.001$), higher complication rates ($p < 0.001$), and higher 28-day mortality rates ($p < 0.001$) (219). Vitamin D deficiency and hypoproteinemia were associated with increased mortality in critically ill patients (219).

Cuñat et al., found that although recommended for all ICU patients, vitamin D was tested in only 17 of the 226 consecutive COVID-19 patients admitted to their hospital in Spain (220). All 17 were vitamin D deficient (25(OH)D <20 ng/ml), 13 had <12.5 ng/ml, and three had <5 ng/ml (220). Of these 17 patients, 35.2% had hypocalcemia and 64.7% had hypophosphatemia (220). The incidence of nosocomial infections was very high (76.5%) (220). The authors stated that vitamin D deficiency is especially problematic for COVID-19

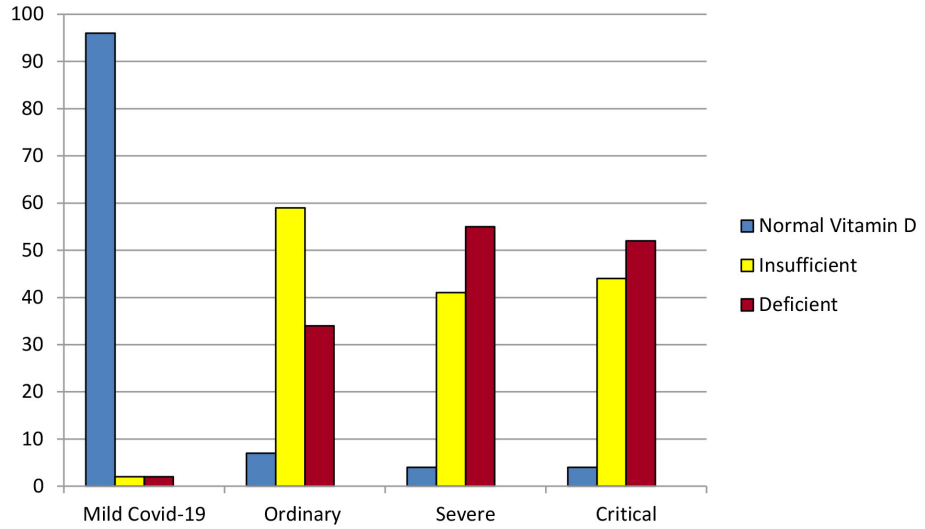


FIGURE 5 | Some of the results of the retrospective chart review by Alipio et al. (3). Of the 212 hospitalized COVID-19 patients, 96% of those with mild COVID-19 had normal vitamin D levels (above 30 ng/ml). In contrast, over 50% of the patients with severe or critical COVID-19 were vitamin D deficient (level 20 ng/ml or lower).

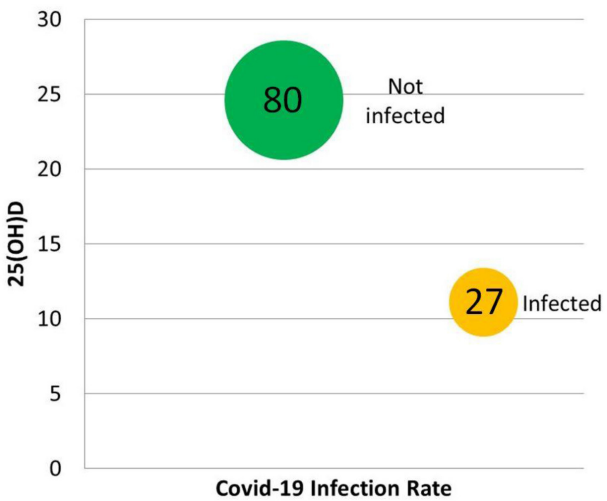


FIGURE 6 | Some of the results of the retrospective chart review by D'Avolio et al. (124). In the 107 individuals tested for both 25(OH)D and COVID-19, vitamin D predicted infection.

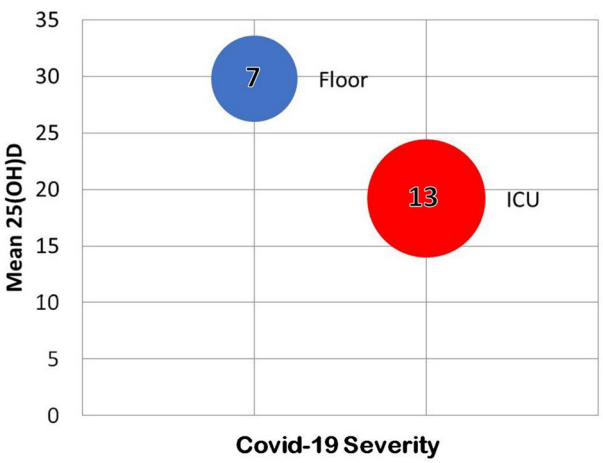


FIGURE 7 | Some of the results of the retrospective chart review by Lau et al. (157). In the 20 hospitalized individuals tested for both 25(OH)D and COVID-19, vitamin D predicted severity of infection.

ICU patients because vitamin D reduces pro-inflammatory and increases anti-inflammatory cytokines (220).

Raharusuna et al., conducted a retrospective chart review of 780 hospitalized test-confirmed COVID-19 patients in Indonesia (4). After controlling for age, sex, and comorbidity, both insufficient (odds ratio 7.63) and deficient vitamin D (odds ratio 10.12) were significantly associated with COVID-19 mortality ($p < 0.001$ for each) (4). Fatalities were 4.1% in patients with normal 25(OH)D, 87.8% with insufficiency, and 98.9% with deficiency (4).

In India, Glicio et al., performed a statistical analysis on the data from the 176 COVID-19 patients 60 years or older in two tertiary medical centers whose medical records included body mass index (BMI), sex, comorbidities, clinical characteristics, and pre-hospitalization 25(OH)D (5). Over 80% were vitamin D insufficient or deficient, and of those, 72% were male (5). Inadequate 25(OH)D was strongly associated with chronic kidney disease, hypertension, and diabetes (5). Vitamin D levels were lower, with a linear distribution, in older patients (oldest age was 85) (5). Insufficient 25(OH)D was found in 45% of the 24 patients with mild COVID-19 vs. 86% of the 131 patients with severe outcomes (5). As age increased, vitamin D levels

correlated linearly with outcomes, with patients over 70 suffering severe COVID-19 only if they were vitamin D insufficient (5). In contrast with obese patients, those with healthy BMIs tended to have severe COVID-19 only if they were vitamin D deficient (also a linear correlation) (**Figure 7**) (5).

De Smet et al., found endemic vitamin D deficiency in their area of Belgium, with lower mean levels in men than women except in summer ($p < 0.05$) (221). Children under age 18 had lower deficiency rates ($p < 0.05$) (221). Comparing 186 consecutive test-positive COVID-19 patients (109 male) with the 2,717 consecutive age-matched controls whose 25(OH)D was tested during the same season in 2019, they found that vitamin D deficiency was prevalent in controls (45.2%), but significantly ($p < 0.05$) more common in the hospitalized COVID-19 patients (58.6%) (221). The median 25(OH)D for COVID-19 patients was 18.6 ng/ml, compared with 21.5 ng/ml for controls ($p = 0.0016$) (221). Male patients were more likely than their control counterparts to be deficient (67.0 vs. 49.2%, $p = 0.0006$) (221). Vitamin D deficiency was strongly associated with more severe COVID-19 pneumonia in males (55.2% with stage 1, 66.7% with stage 2, and 74% with stage 3, $p = 0.001$), but not in females (221). Vitamin D was stable across all stages of COVID-19 for females, suggesting that the illness itself does not deplete vitamin D (221). The authors argue that as a whole, their data supports a causal role for vitamin D deficiency in COVID-19 (221).

Meltzer et al., analyzed data from their US facility's COVID-19 positive patients with documented 25(OH)D levels in EPIC within the previous 2 years to determine if deficiency increases COVID-19 incidence (222). Data for the most recent 25(OH)D and treatment (dose and time span) led to four categories (1) likely still deficient, (2) likely sufficient, (3) likely deficient but improved since testing, and (4) uncertain status (222). Known risk factors and factors that influence vitamin D activation were evaluated (222). A multivariate analysis found that, of patients with 25(OH)D levels within the previous year, those likely to still be deficient (category 1) were more likely (RR = 1.77, $p < 0.02$) to test positive for COVID-19 than those likely to be vitamin D sufficient (category 2) (222) Older age, non-white race, and immunosuppression were the only other factors associated with testing positive for COVID-19 (222). Hypertension, obesity, and diabetes were not covariates with vitamin D (222). Vitamin D deficiency was associated with supplement type and dose ($p < 0.01$), unless the relatively few patients receiving 2,000 IU or more of vitamin D_3 were omitted (indicating that lower doses, D_2, and calcitrol did not improve deficiency) (222). The authors concluded that the relatively low doses of vitamin D usually given to correct deficiency in their institution decreased the apparent benefit of supplementation on COVID-19 rates, and that 4,000–5,000 IU/day may be indicated for COVID-19 prevention (222).

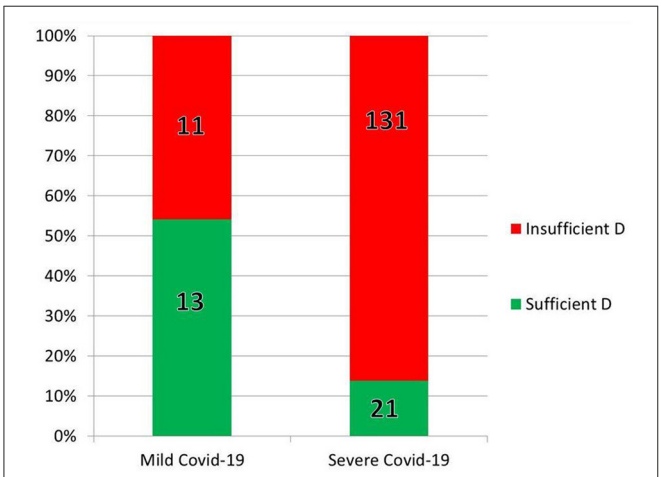


FIGURE 8 | Some of the results of the retrospective chart review by Glicio et al. (5). In the 176 elderly (over age 60) hospitalized individuals tested for both 25(OH)D and COVID-19, vitamin D predicted severity of infection.

Retrospective Chart Reviews That Are Neutral or Strongly Oppose the Hypothesis

Fox and Sizemore evaluated the Electronic Health Records of over 15,000,000 patients in EPIC across 26 US states, finding 28,185 patients with documented 25(OH)D (of which, 86% were deficient) and a documented COVID-19 test (85). No association was found between vitamin D deficiency (defined by each lab) and COVID-19 rates, hospitalizations, or fatalities (85). In contrast with the study by Meltzer et al., no date limits were placed on the testing; the authors noted that vitamin D levels are usually drawn to confirm suspected deficiency (85). The authors recognized this limitation and recommended future studies including patients with normal vitamin D levels, along with studies to assess the effect of vitamin D supplementation on prevention or treatment of COVID-19 (85).

Hastie et al., evaluated data from the 1,474 participants in the UK Biobank study whose COVID-19 test results were available to them (13). Rather than comparing the 1,025 PCR-negative participants to the 449 PCR-positive patients, every person in the 348,598 database without a PCR-positive test result was assumed negative (13). The 25(OH)D levels obtained 10–14 years prior were significantly lower in blacks and South Asians (13). Black or South Asian ethnicity was also strongly associated ($p < 0.001$) with confirmed COVID-19 infection (13). Median 25(OH)D was significantly lower ($p = 0.013$). for those with confirmed COVID-19 infection, and 25(OH)D predicted infection univariably (13). In contrast, the multivariate analysis did not find 25(OH)D was significant (13). Unlike most other studies of COVID-19, the authors found no association between diabetes or hypertension and COVID-19 risk, raising concerns that important variables were factored out in their analysis (13, 84).

Another review using 2006–2010 data from the UK Biobank was conducted by Darling, et al., who compared the vitamin D status, BMI, ethnicity, and lifestyle factors of 580 COVID-19 positive cases (including outpatients) with 723 negative controls of similar age (76). 25(OH)D levels were 3.6 ng/ml lower ($p < 0.001$) in patients who were obese and 6.4 ng/ml lower for those whose ethnicity was not white ($p < 0.001$) (76). COVID-19 risk was increased for non-smokers, London dwellers, males, and non-whites (76). After factoring out overweight and obesity (the factor with the highest odds ratio), and after grouping participant

data into quartiles rather than using individual data, 25(OH)D did not independently predict COVID-19 risk (76).

Raisi-Estabragh et al., conducted a third multivariate analysis on the UK Biobank participants, including all 4,510 who had positive (1,326) or negative (3,184) COVID-19 tests from 16 March to 18 May 2020, almost all of whom were hospitalized (77). The researchers used the baseline data from 10 to 14 years ago for age, sex, deprivation, BMI, and 25(OH)D levels, adjusting the 25(OH)D levels for seasonality and ethnicity (77). Compared with the 497,996 untested participants of the UK Biobank study, men and non-white ethnicities were over-represented in the test group, with black ethnicity being 3.5 times more likely to be test positive than the untested cohort (77). Men and whites had higher average 25(OH)D levels than women and non-white ethnicities (77). Evaluating data from males and females independently, statistical significance was reached for males only for non-white ethnicity, more deprivation, and higher BMI (77). For women, in addition to these three factors, lower 25(OH)D, more overcrowding, and greater risk-taking were all statistically significantly related to testing COVID-19 positive (77). Rather than conducting a multivariate analysis on all potential influencers of COVID-19 positivity, Raisi-Estabragh et al., grouped exposures, testing each group against sex, age, and ethnicity, finding no significant association between these three factors, seasonally and ethnically adjusted 25(OH)D levels, and positive COVID-19 status (77). The researchers found that 25(OH)D and COVID-19 status are confounded by ethnicity and BMI (77) Mean 25(OH)D levels for both COVID-19 negative (14.18 ng/ml) and COVID-19 positive (13.55 ng/ml) primarily hospitalized patients were extremely low (77).

Rapid Systematic Review and Meta-Analysis With an Ecological Approach

Ghasemian et al., conducted a formal systematic review of nine studies, with six studies entering into a meta-analyses, and added their own evaluation of the correlation between global vitamin D status and COVID-19 recovery and mortality (70). The meta-analysis revealed that 46.5% of COVID-19 patients were vitamin D deficient and an additional 43.3% were vitamin D insufficient (70). Although their basic evaluation of 51 countries did not find a significant correlation between population vitamin D status and recovery or mortality rates, when latitude was factored in, both mortality rates and recovery rates weakly supported the vitamin D hypothesis (70). The researchers recommended large randomized clinical trials of vitamin D during the "Age of COVID-19" (70).

COVID-19-SPECIFIC RECOMMENDATIONS OF EXPERTS

Although a few recommended only sunshine or 400 IU/day, none of the authors strongly opposed vitamin D supplements during the pandemic. At the extremes, some researchers recommend large bolus doses of vitamin D, or correction of deficiency, primarily for patients who are diagnosed with COVID-19, and others recommended only the dose of vitamin D needed to maintain bone health (200–400 IU/day) (44, 45, 73, 91, 108, 118, 160, 169, 223–229). Additional authors recommend vitamin D supplements to boost the immune systems of patients diagnosed with COVID-19 (2^p, 35, 69, 71, 74, 75, 118, 120, 125, 230–233). However, most authors recommend widespread daily vitamin D supplementation (most often with 1,000–5,000 IU per day) to prevent and decrease the severity of COVID-19, at least until the pandemic abates (1, 5, 12, 28, 34, 36, 42, 64, 65, 107, 109, 113, 114, 116, 121, 124, 127, 165, 171, 172, 178, 190, 192, 197, 198, 210, 214, 219, 222, 234–248) (**Figure 8**).

Although vitamin D toxicity is extremely rare, considering the recent spate of chloroquine overdoses due to panic from COVID-19, recommendations include cautioning the public that excessive artificial supplementation can lead to serious harm (94, 225, 249, 250). Suresh noted that in India, vitamin D deficiency is due in large part to calcium deficiency, which must therefore also be addressed (234).

Serum response to vitamin D supplementation is highly variable between individuals, leading to recommendations of higher doses than the US RDA (28, 30). The NIH states that vitamin D supplements of up to 5,000 IU/day have not produced toxicity, leading to a maximum recommended intake for persons 9 years and older of 4,000 IU (100 mcg)/day (125, 250). Although the USRDA for vitamin D is 600–800 IU/day, the Endocrine Society and many other experts recommend 1,000–2,000 IU/day (widely available dosages) (31, 64, 65, 250). A comprehensive article on optimizing nutrition to protect against COVID-19 specifically suggests adults take 2,000 IU/day of supplemental vitamin D, in keeping with the recommendations of the US National Academy of Medicine (36, 251). The consensus of the authors reviewed here seems to be 2,000 IU/day for the entire adolescent and adult population.

DISCUSSION

Prior to modern times, individuals living in high latitudes had a much larger food supply from April to October, leading to weight gain (252). Excess vitamin D from sunshine was stored in accumulated fat (24, 206). Weight loss during relatively dark, food-scarce winters, released this excess vitamin D, preserving immune function (24, 206). Now, food is plentiful year-round, leading to weight gain from decreased activity in winter (252). Without weight-loss related vitamin D release, dangerously low 25(OH)D can develop by spring, and the obese, the elderly, those with naturally melanin-rich skin living outside the tropics, and anyone not spending time in the sun are at risk year-round (206).

Sunscreen with a rating of only 15 SPF decreases vitamin D production in the skin by 99% (206). Studies show that non-burning sun exposure increases vitamin D levels and may be melanoma-protective (37). In tropical areas with wealthier populations, sun exposure may decrease in the summer due to a preference for air conditioning (253). Encouraging uninfected people, including the homeless, to stay indoors could cause an increase in COVID-19 fatalities by increasing vitamin D deficiency rates. In contrast, encouraging weight loss through increased activity and structured programs can serve to improve

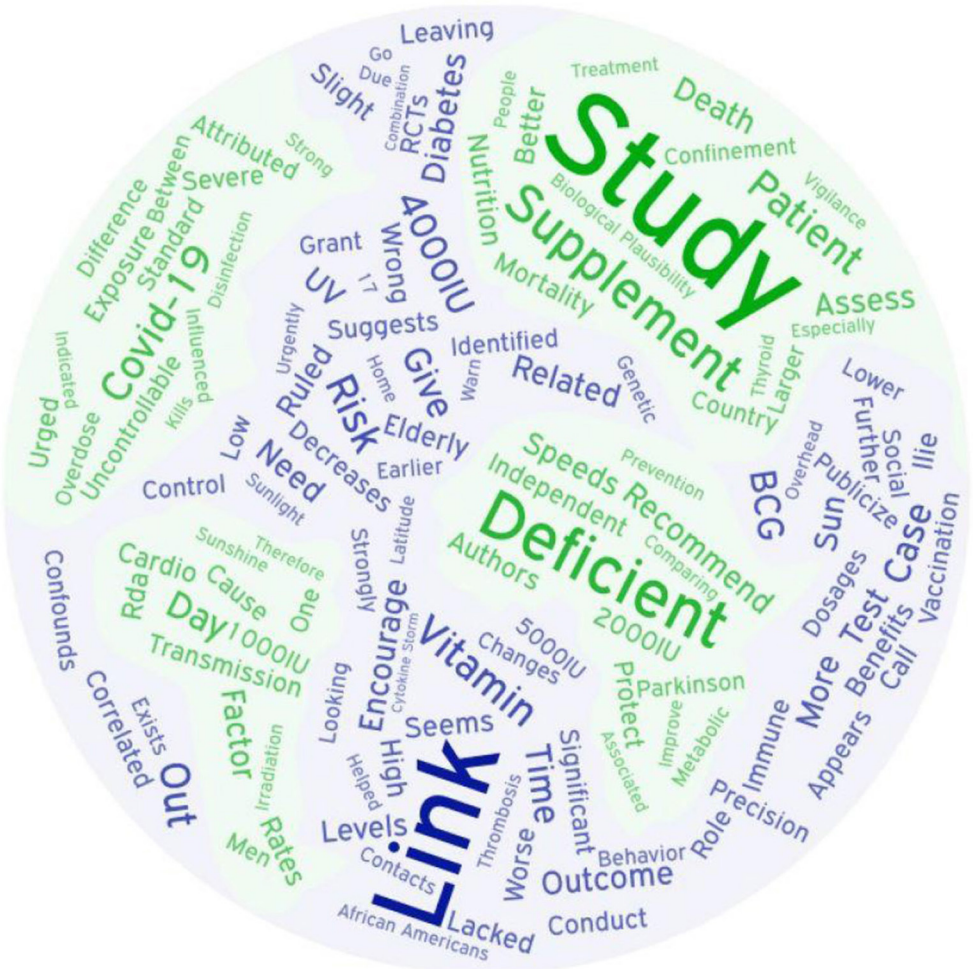


FIGURE 9 | Word Cloud of recommendations from authors of 47 studies.

vitamin D levels (206). Studies show that exercise increases serum vitamin D levels, even when indoors, perhaps by triggering release of vitamin D stored in fat (254).

CONCLUSION

The 141 articles (**Table A1**) presenting primarily biological plausibility evidence overwhelmingly support the assertions that vitamin D sufficiency increases resistance to viral infections and helps prevent every symptom of severe COVID-19 that results in fatalities. They show that vitamin D deficiency can also explain every major risk factor, including the mystery of why children seem relatively protected and why males, the elderly, and people with naturally melanin-rich skin are especially vulnerable.

The 47 studies (**Table A2**) summarized here demonstrate that vitamin D deficiency explains the geographical differences in COVID-19 case and fatality rates. They provide overwhelming correlational evidence for the hypothesis, and causal evidence as well. COVID-19 mortality was predicted by vitamin D in 16 studies (4, 14, 24, 27, 63, 122, 127, 146, 150, 182, 197, 206, 209–211, 219) and vitamin D levels or sunlight predicted contracting COVID-19 in 17 (34, 122, 124, 150, 197, 198, 200, 201, 204, 205, 207–209, 211, 216, 221, 222). Both causal modeling studies and eight chart reviews demonstrated that lower 25(OH)D was linearly associated with more severe COVID-19 outcomes (3–5, 63, 157, 211, 215, 217, 219, 221).

None of the four objections to recommending universal vitamin D supplements are supported by the evidence. The exhaustive literature search found no vitamin D proponent who suggested that COVID-19 could be completely eliminated with supplementation. Rather than overstating the case, they present compelling evidence that vitamin D deficiency is one factor which increases risk for COVID-19 infection and progression. Although overdoses are theoretically possible, they are highly improbable. The recommended dose by consensus, 2,000 IU/day for adults, is 1/20th the amount that must be taken for many months to risk toxicity (28, 88, 91, 92). The evidence strongly suggests that vitamin D deficiency is an easily modifiable risk factor and correcting it is potentially life-saving. Suppressing this

evidence out of fear that the public might believe supplements will make them "immune" to COVID-19 is not only elitist, but it is inconsistent with existing public policy approaches. Many mitigation strategies are publicized. None are seen as conferring immunity.

This succinct but comprehensive review of the evidence found that despite almost complete absence of official government guidelines favoring vitamin D supplements to potentially decrease COVID-19 risk and severity, support among clinicians and other researchers for correcting and preventing vitamin D deficiency with modest daily vitamin D supplementation during the COVID-19 pandemic is very strong, worldwide. The evidence supports recommending 2,000 IU (50 mcg) vitamin D daily for at-risk teens and adults, which is well within safe limits and might dramatically reduce COVID-19 fatalities.

LIMITATIONS

Many of the articles and studies included in this review were preprints, or were published in haste. The study descriptions were often too brief for a critical appraisal of the designs. Definitions of variables, such as race and ethnicity, were often omitted. Many researchers did not make their data public, although some emailed corrections or clarifications. Although the author is familiar with inflammation and cytokines from her work with chronic wounds, and she is familiar with epidemiology from her health education work in developing countries, she is not an endocrinologist or an epidemiologist. Single authorship could also be considered a limitation.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR'S NOTE

Linda Benskin is an independent nurse researcher working to improve the evidence base for village health workers in remote and conflict areas of tropical developing countries, where health care professionals are absent. Her research into how pain and inflammation impact wound healing has provided her with a basic familiarity with cytokine pathophysiology. Dr. Benskin's improvised wound dressings clinical research study has been sidelined by the travel restrictions of COVID-19. Dr. Benskin is also the Clinical Research, Education, & Charity Liaison for Ferris Mfg. Corp. (makers of PolyMem dressings).

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

ACKNOWLEDGMENTS

The author wishes to thank her research associate, Richard Benskin, who provided invaluable editing help, John Newton whose encouragement led to this review, and her employer, Ferris Mfg. Corp., makers of PolyMem, who generously provided her with the time, as their charity liaison, to conduct the literature search and write this review.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2020.00513/full#supplementary-material

REFERENCES

1. Wang L, Wang Y, Ye D, Liu Q. Review of the 2019 novel coronavirus (SARS-CoV-2) based on current evidence. *Int J Antimicrobial Agents.* (2020) 55:105948. doi: 10.1016/j.ijantimicag.2020.105948
2. Bloukh SH, Shaikh AA, Pathan HM, Edis Z. Prevalence of COVID-19: a Look behind the Scenes. *Preprint*. (2020). doi: 10.20944/preprints202004.0179.v2
3. Alipio M. *Vitamin D Supplementation Could Possibly Improve Clinical Outcomes of Patients Infected with Coronavirus-2019 (COVID-2019)*. Rochester, NY: Social Science Research Network (2020). doi: 10.2139/ssrn.3571484
4. Raharusun P, Priambada S, Budiarti C, Agung E, Budi C. *Patterns of COVID-19 Mortality and Vitamin D: An Indonesian Study*. Emerginnova (2020). Available online at: https://emerginnova.com/patterns-of-covid19-mortality-and-vitamin-d-an-indonesian-study/ (accessed May 20, 2020).
5. Glicio EJ. *Vitamin D Level of Mild and Severe Elderly Cases of COVID-19: A Preliminary Report*. Rochester, NY: Social Science Research Network (2020). Available online at: https://papers.ssrn.com/abstract=3593258 (accessed May 20, 2020).
6. Chinese CDC. *The Epidemiological Characteristics of an Outbreak of 2019 Novel Coronavirus Diseases (COVID-19) — China, 2020*. Available online at: http://weekly.chinacdc.cn/en/article/id/e53946e2-c6c4-41e9-9a9b-fea8db1a8f51 (accessed April 23, 2020).
7. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet.* (2020) 395:497–506. doi: 10.1016/S0140-6736(20)30183-5
8. Lighter J, Phillips M, Hochman S, Sterling S, Johnson D, Francois F, et al. Obesity in patients younger than 60 years is a risk factor for Covid-19 hospital admission. *Clin Infect Dis.* (2020) 71:896–7. doi: 10.1093/cid/ciaa415
9. Simonnet A, Chetboun M, Poissy J, Raverdy V, Noulette J, Duhamel A, et al. High prevalence of obesity in severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) requiring invasive mechanical ventilation. *Obesity.* (2020) 28:1195–9. doi: 10.1002/oby.22831
10. Brown RA. Re: preventing a covid-19 pandemic - COVID-19: vitamin D deficiency; and, death rates; are both disproportionately higher in elderly Italians, Spanish, Swedish Somali, and African Americans? A connection? Research urgently required! *BMJ*. (2020). Available online at: https://www.bmj.com/content/368/bmj.m810/rr-46 (accessed April 23, 2020).
11. Docherty AB, Harrison EM, Green CA, Hardwick HE, Pius R, Norman L, et al. Features of 20 133 UK patients in hospital with covid-19 using the

ISARIC WHO clinical characterisation protocol: prospective observational cohort study. *BMJ.* (2020) 369:m1985. doi: 10.1136/bmj.m1985
12. Ebadi M, Montano-Loza AJ. Perspective: improving vitamin D status in the management of COVID-19. *Eur J Clin Nutr.* (2020) 74:856–9. doi: 10.1038/s41430-020-0661-0
13. Hastie CE, Mackay DF, Ho F, Celis-Morales CA, Katikireddi SV, Niedzwiedz CL, et al. Vitamin D concentrations and COVID-19 infection in UK Biobank. *Diabetes Metab Syndrome Clin Res Rev.* (2020) 14:561–5. doi: 10.1016/j.dsx.2020.04.050
14. Bäcker A. Why COVID-19 may be disproportionately killing African Americans: black overrepresentation among COVID-19 mortality increases with lower irradiance, where ethnicity is more predictive of COVID-19 infection and mortality than median income. *Soc Sci Res Netw.* (2020). doi: 10.2139/ssrn.3571699
15. Kass DA, Duggal P, Cingolani O. Obesity could shift severe COVID-19 disease to younger ages. *Lancet.* (2020) 395:1544–5. doi: 10.1016/S0140-6736(20)31024-2
16. Cook T, Kursumovic E, Lennane S. Exclusive: deaths of NHS staff from covid-19 analysed. *Health Serv J.* (2020). Available online at: https://www.hsj.co.uk/exclusive-deaths-of-nhs-staff-from-covid-19-analysed/7027471. article (accessed June 24, 2020).
17. Gudbjartsson DF, Helgason A, Jonsson H, Magnusson OT, Melsted P, Norddahl GL, et al. Spread of SARS-CoV-2 in the Icelandic Population. *N Engl J Med.* (2020) 382:2302–15. doi: 10.1056/NEJMoa2006100
18. National Centre for Immunisation Research and Surviillience. *COVID-19 in Schools – the Experience in NSW | NCIRS*. National Centre for Immunisation Research and Surviillience (2020). Available online at: http://www.ncirs.org.au/covid-19-in-schools (accessed May 21, 2020).
19. Grant WB, Lahore H, McDonnell SL, Baggerly CA, French CB, Aliano JL, et al. Evidence that vitamin d supplementation could reduce risk of influenza and COVID-19 infections and deaths. *Nutrients.* (2020) 12:988. doi: 10.3390/nu12040988
20. Conticini E, Frediani B, Caro D. Can atmospheric pollution be considered a co-factor in extremely high level of SARS-CoV-2 lethality in Northern Italy? *Environ Pollut.* (2020) 261:114465. doi: 10.1016/j.envpol.2020.114465
21. Martelletti L, Martelletti P. Air pollution and the novel Covid-19 disease: a putative disease risk factor. *SN Compr Clin Med.* (2020). doi: 10.1007/s42399-020-00274-4. [Epub ahead of print].
22. Zhu Y, Xie J, Huang F, Cao L. Association between short-term exposure to air pollution and COVID-19 infection: evidence from China. *Sci Total Environ.* (2020) 727:138704. doi: 10.1016/j.scitotenv.2020.138704
23. Carleton T, Meng KC. Causal empirical estimates suggest COVID-19 transmission rates are highly seasonal. *medRxiv.* (2020) 2020.03.26.20044420. doi: 10.1101/2020.03.26.20044420
24. Moozhipurath RK, Kraft L, Skiera B. Evidence of protective role of ultraviolet-B (UVB) radiation in reducing COVID-19 deaths. *medRxiv.* (2020) 2020.05.06.20093419. doi: 10.1101/2020.05.06.20093419
25. Cao Z, Wu Y, Faucon E, Sabatier J-M. SARS-CoV-2 & Covid-19: key-roles of the "renin-angiotensin" system / vitamin D impacting drug and vaccine developments. *Infect Disord Drug Targets.* (2020) 20:348–9. doi: 10.2174/1871526520999200505174704
26. Rhodes JM, Subramanian S, Laird E, Anne Kenny R. Editorial: low population mortality from COVID-19 in countries south of latitude 35 degrees North - supports vitamin D as a factor determining severity. *Aliment Pharmacol Ther.* (2020) 51:1434–7. doi: 10.1111/apt.15777
27. Laird E, Rhodes J, Kenny RA. Vitamin D and Inflammation – Potential Implications for Severity of Covid-19. *Ir Med J.* (2020) 113:81.
28. Hribar CA, Cobbold PH, Church FC. Potential role of vitamin D in the Elderly to resist COVID-19 and to slow progression of Parkinson's disease. *Brain Sci.* (2020) 10:284. doi: 10.3390/brainsci10050284
29. Holick MF. Vitamin D status: measurement, interpretation and clinical application. *Ann Epidemiol.* (2009) 19:73–8. doi: 10.1016/j.annepidem.2007.12.001
30. Fabbri A, Infante M, Ricordi C. Editorial - Vitamin D status: a key modulator of innate immunity and natural defense from acute viral respiratory infections. *Eur Rev Med Pharmacol Sci.* (2020) 24:4048–52. doi: 10.26355/eurrev_202004_20876
31. Boucher BJ. The problems of vitamin D insufficiency in older people. *Aging Dis.* (2012) 3:313–29.
32. Crane-Godreau MA, Clem KJ, Payne P, Fiering S. Vitamin D deficiency and air pollution exacerbate COVID-19 through suppression of antiviral peptide LL37. *Front Public Health.* (2020) 8:232. doi: 10.3389/fpubh.2020.00232
33. Ghavideldarestani M, Honardoost M, Khamseh ME. Role of vitamin D in pathogenesis and severity of COVID-19 infection. *Preprint.* (2020) doi: 10.20944/preprints202004.0355.v1
34. Kara M, Ekiz T, Ricci V, Kara Ö, Chang K-V, Özçakar L. 'Scientific Strabismus' or two related pandemics: coronavirus disease and vitamin D deficiency. *Br J Nutr.* (2020). doi: 10.1017/S0007114520001749. [Epub ahead of print].
35. Caccialanza R, Laviano A, Lobascio F, Montagna E, Bruno R, Ludovisi Set al. Early nutritional supplementation in non-critically ill patients hospitalized for the 2019 novel coronavirus disease (COVID-19): rationale and feasibility of a shared pragmatic protocol. *Nutrition.* (2020) 74:110835. doi: 10.1016/j.nut.2020.110835
36. Calder PC, Carr AC, Gombart AF, Eggersdorfer M. Optimal nutritional status for a well-functioning immune system is an important factor to protect against viral infections. *Nutrients.* (2020) 12:1181. doi: 10.3390/nu12041181
37. Hoel DG, Berwick M, de Gruijl FR, Holick MF. The risks and benefits of sun exposure 2016. *Dermato Endocrinol.* (2016) 8:e1248325. doi: 10.1080/19381980.2016.1248325
38. La Vignera S, Cannarella R, Condorelli RA, Torre F, Aversa A, Calogero AE. Sex-specific SARS-CoV-2 mortality: among hormone-modulated ACE2 expression, risk of venous thromboembolism and hypovitaminosis D. *Int J Mol Sci.* (2020) 21:2948. doi: 10.3390/ijms21082948
39. McCartney DM, Byrne DG. McCartney et al. comment on 'Covid-19, Cocooning and Vitamin D Requirements' response report. *Ir Med J.* (2020) 113:80
40. Perry T. Tyler Perry Encouraging Blacks To Increase Vitamin D During COVID-19. *Black Enterprise* (2020). Available online at: https://www.blackenterprise.com/tyler-perry-is-encouraging-black-people-to-increase-their-vitamin-d-during-the-covid-19-pandemic/ (accessed May 4, 2020).
41. Illmatical C. *African Americans High COVID-19 Mortality Rates: Is This Due to a Lack of Vitamin D? | Afro*. Available online at: https://www.afro.com/african-americans-high-covid-19-mortality-rates-is-this-due-to-a-lack-of-vitamin-d/ (accessed May 4, 2020).
42. Pinnock D. Vitamin D and COVID-19. The Evidence Warrants Discussion! *The Medicinal Chef Making Health Simple* (2020). Available online at: https://www.themedicinalchef.co.uk/vitamin-d-and-covid-19-the-evidence-warrants-discussion/ (accessed June 25, 2020).
43. Trinity College Dublin. Vitamin D determines severity in COVID-19 so government advice needs to change. *EurekAlert!* Available online at: https://www.eurekalert.org/pub_releases/2020-05/tcd-vdd051220.php (accessed May 14, 2020).
44. Garg M, Al-Ani A, Mitchell H, Hendy P, Christensen B. Editorial: low population mortality from COVID-19 in countries south of latitude 35 degrees North - supports vitamin D as a factor determining severity. Authors' reply. *Aliment Pharmacol Ther.* (2020) 51:1438–9. doi: 10.1111/apt.15796
45. Lee J, van Hecke O, Roberts N. Vitamin D: A rapid review of the evidence for treatment or prevention in COVID-19. *CEBM.* Available online at: https://www.cebm.net/covid-19/vitamin-d-a-rapid-review-of-the-evidence-for-treatment-or-prevention-in-covid-19/ (accessed May 4, 2020).
46. *Should People Take Vitamin D to Ward off the New Coronavirus*? Available online at: https://www.medicalnewstoday.com/articles/should-people-take-vitamin-d-to-ward-off-the-new-coronavirus (accessed May 8, 2020).
47. McRae M. COVID-19 deaths are being linked to vitamin D deficiency. Here's what that means. *ScienceAlert.* Available online at: https://www.sciencealert.com/covid-deaths-are-being-linked-with-vitamin-d-deficiency-here-s-what-that-means (accessed May 8, 2020).
48. Lee BY. Can vitamin D help with Covid-19 coronavirus? Here is the science. *Forbes.* Available online at: https://www.forbes.com/sites/brucelee/2020/05/17/can-vitamin-d-help-with-covid-19-coronavirus-here-is-the-science/ (accessed May 19, 2020).

49. Can Vitamin D Prevent COVID-19? *Health Essentials from Cleveland Clinic* (2020). Available online at: https://health.clevelandclinic.org/can-vitamin-d-prevent-covid-19/ (accessed May 19, 2020).
50. Barron A, Patel N. *Vitamin D: A Rapid Review of the Evidence for Treatment or Prevention of COVID-19 in Adults (Excluding Pregnant & Breastfeeding Women)*. London: NIH (2020). Available online at: https://www.ncl-mon.nhs.uk/faq/guidelines/
51. Auwaerter PG. *UPDATE 5/20/2020 - COVID-19: Keeping Up with a Moving Target*. Johns Hopkins: DKBMed (2020). Available at: https://www.youtube.com/watch?v=LaAQ8rX4x9g (accessed May 24, 2020).
52. Wan DM. In Conversation – Nobel Laureate Prof. Peter Doherty. *SR Vol57(06)* (2020). Available online at: http://nopr.niscair.res.in/handle/123456789/54393 (accessed May 28, 2020).
53. Rabbitt L, Slattery E. Vitamin D and Covid-19 – A note of caution. *Ir Med J.* (2020) 113:82.
54. Chhetri JK, Chan P, Arai H, Chul Park S, Sriyani Gunaratne P, Setiati S, et al. Prevention of COVID-19 in older adults: a brief guidance from the International Association for Gerontology and Geriatrics (IAGG) Asia/Oceania region. *J Nutr Health Aging.* (2020) 24:471–2. doi: 10.1007/s12603-020-1359-7
55. Raymond M, Ching-A-Sue G, Oliver VH. *Mast Cell Stabilisers, Leukotriene Antagonists and Antihistamines: A Rapid Review of Effectiveness in COVID-19*. Centre for Evidence-Based Medicine (2020). Available online at: https://covid19-evidence.paho.org/handle/20.500.12663/1569 (accessed June 16, 2020).
56. Isaia G, Medico E. *Possible Prevention and Therapeutic Role of Vitamin D in the Management of the COVID-19 2020 Pandemics*. (2020). Available online at: https://minapim.com/en/possible-preventive-and-therapeutic-role-of-vitamin-d-in-the-management-of-the-covid-19-pandemic/
57. Ribeiro H, de Santana KV, Oliver SL, de Rondó PHC, Mendes MM, Charlton K, Lanhamet al. Does Vitamin D play a role in the management of Covid-19 in Brazil? *Rev Saúde Públ.* 54. doi: 10.11606/s1518-8787.2020054002545
58. Nota de Esclarecimento: Vitamina D e Covid-19. *Sociedade Brasileira de Endocrinologia e Metabologia* Available online at: https://www.endocrino.org.br/nota-de-esclarecimento-vitamina-d-e-covid-19/ (accessed June 26, 2020).
59. Thebault R, Tran AB, Williams V. The coronavirus is infecting and killing black Americans at an alarmingly high rate. *Washington Post* (2020). Available online at: https://www.washingtonpost.com/nation/2020/04/07/coronavirus-is-infecting-killing-black-americans-an-alarmingly-high-rate-post-analysis-shows/ (accessed May 7, 2020).
60. Eligon J, Burch ADS, Searcey D, Jr RAO. Black Americans face alarming rates of coronavirus infection in some states. *The New York Times* (2020). Available online at: https://www.nytimes.com/2020/04/07/us/coronavirus-race.html (accessed June 28, 2020).
61. CDC. *COVID-19 in Racial and Ethnic Minority Groups*. Centers for Disease Control and Prevention (2020). Available online at: https://www.cdc.gov/coronavirus/2019-ncov/need-extra-precautions/racial-ethnic-minorities.html (accessed June 28, 2020).
62. Wadhera RK, Wadhera P, Gaba P, Figueroa JF, Maddox KEJ, Yeh RW, et al. Variation in COVID-19 hospitalizations and deaths across New York City Boroughs. *JAMA*. (2020) 323:2192–5. doi: 10.1001/jama.2020.7197
63. Davies G, Garami AR, Byers JC. Evidence supports a causal model for vitamin D in COVID-19 outcomes. *medRxiv*. (2020) 2020.05.01.20087965. doi: 10.1101/2020.05.01.20087965
64. Tom Frieden MD. Former CDC Chief Dr. Tom Frieden: Coronavirus infection risk may be reduced by Vitamin D. *Fox News* (2020). Available online at: https://www.foxnews.com/opinion/former-cdc-chief-tom-frieden-coronavirus-risk-may-be-reduced-with-vitamin-d (accessed May 8, 2020).
65. Manson JE. Does Vitamin D Protect Against COVID-19? *Medscape* (2020). Available online at: http://www.medscape.com/viewarticle/930152 (accessed May 12, 2020).
66. Doheny K. More Vitamin D, Lower Risk of Severe COVID-19? *WebMD*. Available online at: https://www.webmd.com/lung/news/20200518/more-vitamin-d-lower-risk-of-severe-covid-19 (accessed May 19, 2020).
67. British Dietetic Association. *COVID-19 / Coronavirus - Advice for the General Public* (2020). Available online at: https://www.bda.uk.com/resource/covid-19-corona-virus-advice-for-the-general-public.html (accessed May 19, 2020).
68. McCall B. Vitamin D: A Low-Hanging Fruit in COVID-19? *Medscape*. Available online at: http://www.medscape.com/viewarticle/930660 (accessed June 15, 2020).
69. Mitchell F. Vitamin-D and COVID-19: do deficient risk a poorer outcome? *Lancet Diabetes Endocrinol.* (2020) 8:570. doi: 10.1016/S2213-8587(20)30183-2
70. Ghasemian R, Shamshirian A, Heydari K, Malekan M, Alizadeh-Navaei R, Ebrahimzadeh MA, et al. The role of vitamin D in the age of COVID-19: a systematic review and meta-analysis along with an ecological approach. *medRxiv*. (2020) 2020.06.05.20123554. doi: 10.1101/2020.06.05.20123554
71. Taheri S, Chagoury O, Tourette M, Skaroni I, Othman M, Bashir M, et al. Managing diabetes in Qatar during the COVID-19 pandemic. *Lancet Diabetes Endocrinol.* (2020) 8:473–4. doi: 10.1016/S2213-8587(20)30154-6
72. Laird E, Kenny RA. Vitamin D deficiency in Ireland – implications for COVID-19. Results from the Irish Longitudinal Study on Ageing (TILDA). The Irish Longitudinal Study on Ageing (2020). doi: 10.38018/TildaRe.2020-05
73. McKenna MJ, Flynn MAT. Covid-19 cocooning and vitamin D intake requirements. *Ir Med J.* (2020) 113:79.
74. Molloy EJ, Murphy N. Vitamin D, Covid-19 and children. *Ir Med J.* (2020) 113:64.
75. McCartney DM, Byrne DG. Optimisation of vitamin D status for enhanced immuno-protection against Covid-19. *Ir Med J.* (2020) 113:58.
76. Darling AL, Ahmadi KR, Ward KA, Harvey NC, Alves AC, Dunn-Waters DK, et al. Vitamin D status, body mass index, ethnicity and COVID-19: initial analysis of the first-reported UK Biobank COVID-19 positive cases (n 580) compared with negative controls (n 723). *medRxiv*. (2020) 2020.04.29.20084277. doi: 10.1101/2020.04.29.20084277
77. Raisi-Estabragh Z, McCracken C, Bethell MS, Cooper J, Cooper C, Caulfield MJ, et al. Greater risk of severe COVID-19 in non-White ethnicities is not explained by cardiometabolic, socioeconomic or behavioural factors, or by 25(OH)-vitamin D status: study of 1,326 cases from the UK Biobank. *J Public Health.* 42, 451–46 (2020). doi: 10.1093/pubmed/fdaa095/5859581. [Epub ahead of print].
78. Meng JE, Hovey KM, Wactawski-Wende J, Andrews CA, LaMonte MJ, Horst RL, et al. Intraindividual variation in plasma 25-Hydroxyvitamin D measures 5 years apart among postmenopausal women. *Cancer Epidemiol Biomarkers Prev.* (2012) 21:916–24. doi: 10.1158/1055-9965.EPI-12-0026
79. Roy AS, Matson M, Herlekar R. Response to 'Vitamin D concentrations and COVID-19 infection in UK Biobank.' *Diabetes Metab Syndrome Clin Res Rev.* (2020) 14:777. doi: 10.1016/j.dsx.2020.05.049
80. Etsy E. *The Possible Link Between Severe COVID-19 and Low Vitamin D Levels*. Available online at: https://www.youtube.com/watch?v=_ZJkDvVFbRE (accessed June 22, 2020).
81. Jorde R, Sneve M, Hutchinson M, Emaus N, Figenschau Y, Grimnes G. Tracking of serum 25-hydroxyvitamin D levels during 14 years in a population-based study and during 12 months in an intervention study. *Am J Epidemiol.* (2010) 171:903–8. doi: 10.1093/aje/kwq005
82. Yong SJ. Population studies confirm risk factors for catching Covid-19. *Medium* (2020). Available online at: https://medium.com/microbial-instincts/population-studies-confirm-risk-factors-for-catching-covid-19-853779e7a3c6 (accessed June 21, 2020).
83. Boucher BJ. Adjustments in analyses of vitamin D status, allowing for vitamin D determinants, for Covid-19 risks. *Diabetes Metab Res Rev.* (2020) e3375. doi: 10.1002/dmrr.3375
84. Grant WB, McDonnell SL. Letter in response to the article: vitamin D concentrations and COVID-19 infection in UK biobank (Hastie et al.). *Diabetes Metab Syndrome Clin Res Rev.* (2020) 14:893–4. doi: 10.1016/j.dsx.2020.05.046
85. Fox B, Sizemore JO. No association seen between vitamin D deficiency and COVID-19 infection, hospitalization, or mortality. *Epic Health Res. Netw* (2020). Available online at: http://ehrn.org/no-association-seen-between-vitamin-d-deficiency-and-covid-19-infection-hospitalization-or-mortality/

86. DeFilipps K. Comment on No Association Seen Between Vitamin D Deficiency and COVID-19 Infection, Hospitalization, or Mortality. *Epic Health Research Network* (2020). Available online at: http://ehrn.org/no-association-seen-between-vitamin-d-deficiency-and-covid-19-infection-hospitalization-or-mortality/ (accessed June 23, 2020).
87. Christakos S, Li S, De La Cruz J, Bikle DD. New developments in our understanding of vitamin D metabolism, action and treatment. *Metabolism.* (2019) 98:112–20. doi: 10.1016/j.metabol.2019.06.010
88. Marcinowska-Suchowierska E, Kupisz-Urbańska M, Łukaszkiewicz J, Płudowski P, Jones G. Vitamin D toxicity–A clinical perspective. *Front Endocrinol.* (2018) 9:550. doi: 10.3389/fendo.2018.00550
89. Solis M. A cloud of doubt over the sunshine vitamin. *Pharm J.* (2020) 304. doi: 10.1211/PJ.2020.20207521
90. Buttriss JL, Lanham-New SA. Is a vitamin D fortification strategy needed? *Nutr Bull.* (2020) 45:115–22. doi: 10.1111/nbu.12430
91. Bakare TA, Soar JS. A cost-effective preventative approach to potentially save lives in the coronavirus pandemic, jointly using Vitamin D, Curcumin, and Vitamin C. *IndiaRxiv Preprints* (2020). Available online at: https://indiarxiv.org/cq8sa/ (accessed May 8, 2020).
92. Meehan M, Penckofer S. The role of vitamin D in the aging adult. *J Aging Gerontol.* (2014) 2:60–71. doi: 10.12974/2309-6128.2014.02.02.1
93. Kimball SM, Mirhosseini N, Holick MF. Evaluation of vitamin D3 intakes up to 15,000 international units/day and serum 25-hydroxyvitamin D concentrations up to 300 nmol/L on calcium metabolism in a community setting. *Dermatoendocrinol.* (2017) 9:e1300213. doi: 10.1080/19381980.2017.1300213
94. Lim K, Thadhani R, Lim K, Thadhani R. Vitamin D Toxicity. *Braz J Nephrol.* (2020) 42:238–44. doi: 10.1590/2175-8239-jbn-2019-0192
95. Spiller HA, Good TF, Spiller NE, Aleguas A. Vitamin D exposures reported to US poison centers 2000-2014: temporal trends and outcomes. *Hum Exp Toxicol.* (2016) 35:457–61. doi: 10.1177/0960327115595685
96. Veugelers P, Ekwaru J. A statistical error in the estimation of the recommended dietary allowance for vitamin D. *Nutrients.* (2014) 6:4472–5. doi: 10.3390/nu6104472
97. Heaney R, Garland C, Baggerly C, French C, Gorham E. Letter to Veugelers, P.J. and Ekwaru, J.P., A statistical error in the estimation of the recommended dietary allowance for vitamin D. Nutrients 2014, 6, 4472-4475; doi: 10.3390/nu6104472. *Nutrients.* (2015) 7:1688–90. doi: 10.3390/nu7031688
98. Burt LA, Billington EO, Rose MS, Raymond DA, Hanley DA, Boyd SK. Effect of high-dose vitamin D supplementation on volumetric bone density and bone strength: a randomized clinical trial. *JAMA.* (2019) 322:736–45. doi: 10.1001/jama.2019.11889
99. McCullough PJ, Amend J, McCullough WP, Repas SJ, Travers JB, Lehrer DS. The essential role of vitamin D in the biosynthesis of endogenous antimicrobial peptides may explain why deficiency increases mortality risk in COVID-19 infections. *Preprint.* (2020) doi: 10.20944/preprints202005.0265.v1
100. Singer P, Blaser AR, Berger MM, Alhazzani W, Calder PC, Casaer MP, et al. ESPEN guideline on clinical nutrition in the intensive care unit. *Clin Nutr.* (2019) 38:48–79. doi: 10.1016/j.clnu.2018.08.037
101. Early high-dose vitamin D3 for critically Ill, vitamin D–deficient patients. *N Engl J Med.* (2019) 381:2529–40. doi: 10.1056/NEJMoa1911124
102. Han JE, Jones JL, Tangpricha V, Brown MA, Hao L, Hebbar G, et al. High dose vitamin D administration in ventilated intensive care unit patients: a pilot double blind randomized controlled trial. *J Clin Transl Endocrinol.* (2016) 4:59–65. doi: 10.1016/j.jcte.2016.04.004
103. Kow CS, Hadi MA, Hasan SS. Vitamin D supplementation in influenza and COVID-19 infections comment on: "evidence that vitamin D supplementation could reduce risk of influenza and COVID-19 infections and deaths" nutrients 2020, 12(4), 988. *Nutrients.* (2020) 12:1626. doi: 10.3390/nu12061626
104. Martineau AR, Jolliffe DA, Hooper RL, Greenberg L, Aloia JF, Bergman P, et al. Vitamin D supplementation to prevent acute respiratory tract infections: systematic review and meta-analysis of individual participant data. *BMJ.* (2017) 356:i6583. doi: 10.1136/bmj.i6583
105. Grant WB, Baggerly CA, Lahore H. Reply: "Vitamin D supplementation in influenza and COVID-19 infections. Comment on: evidence that vitamin D supplementation could reduce risk of influenza and COVID-19 infections and deaths nutrients 2020, 12(4), 988." *Nutrients.* (2020) 12:1620. doi: 10.3390/nu12061620
106. Aloia JF, Li-Ng M. Re: epidemic influenza and vitamin D. *Epidemiol Infect.* (2007) 135:1095–6; author reply 1097–8. doi: 10.1017/S0950268807008308
107. Sharma SK, Mudgal SK, Pai VS, Chaturvedi J, Gaur R. Vitamin D: a cheap yet effective bullet against coronavirus disease-19–Are we convinced yet? *Natl J Physiol Pharm Pharmacol.* (2020) 10. doi: 10.5455/njppp.2020.10.05132202024052020
108. Quesada-Gomez JM, Castillo ME, Bouillon R. Vitamin D Receptor stimulation to reduce Acute Respiratory Distress Syndrome (ARDS) in patients with Coronavirus SARS-CoV-2 infections: revised Ms SBMB 2020_166. *J Steroid Biochem Mol Biol.* (2020) 202:105719. doi: 10.1016/j.jsbmb.2020.105719
109. Chakhtoura M, Napoli N, El Hajj Fuleihan G. Commentary: myths and facts on vitamin D amidst the COVID-19 pandemic. *Metabolism.* (2020) 109:154276. doi: 10.1016/j.metabol.2020.154276
110. Kakodkar P, Kaka N, Baig MN. A comprehensive literature review on the clinical presentation, and management of the pandemic coronavirus disease 2019 (COVID-19). *Cureus.* (2020) 12:e7560. doi: 10.7759/cureus.7560
111. Bokharee N, Khan YH, Wasim T, Mallhi TH, Alotaibi NH, Iqbal MS, et al. Daily versus stat vitamin D supplementation during pregnancy; A prospective cohort study. *PLoS ONE.* (2020) 15:e0231590. doi: 10.1371/journal.pone.0231590
112. Brown RA, Sarkar A. Vitamin D deficiency: a factor in COVID-19, progression, severity and mortality? – An urgent call for research. *MitoFit Preprint Arch.* (2020). doi: 10.26124/mitofit:200001
113. Carter SJ, Baranauskas MN, Fly AD. Considerations for obesity, vitamin D, and physical activity amidst the COVID-19 pandemic. *Obesity.* (2020) 28:1176–7. doi: 10.1002/oby.22838
114. Tian Y, Rong L. Letter: covid-19 and vitamin D-authors' reply. *Aliment Pharmacol Ther.* (2020) 51:995–6. doi: 10.1111/apt.15764
115. Jakovac H. COVID-19 and vitamin D—Is there a link and an opportunity for intervention? *Am J Physiol Endocrinol Metabol.* (2020) 318:E589. doi: 10.1152/ajpendo.00138.2020
116. Misra DP, Agarwal V, Gasparyan AY, Zimba O. Rheumatologists' perspective on coronavirus disease 19 (COVID-19) and potential therapeutic targets. *Clin Rheumatol.* (2020) 39:2055–62. doi: 10.1007/s10067-020-05073-9
117. Turashvili N, Javashvili L. Could vitamin D reduce the risk of COVID-19? *Transl Clin Med Georgian Med J.* (2020) 5:4–6.
118. Parvin F, Islam S, Urmy Z, Ahmed S. The symptoms, contagious process, prevention and post treatment of COVID-19. *Eur J Physiother Rehabil Stud.* (2020). Available at: https://oapub.org/hlt/index.php/EJPRS/article/view/52 (accessed May 8, 2020).
119. Islam MR, Fischer A. A Transcriptome analysis identifies potential preventive and therapeutic approaches towards COVID-19. *Preprint.* (2020) doi: 10.20944/preprints202004.0399.v1
120. Zhang L, Liu Y. Potential interventions for novel coronavirus in China: a systematic review. *J Med Virol.* (2020) 92:479–90. doi: 10.1002/jmv.25707
121. Jayawardena R, Sooriyaarachchi P, Chourdakis M, Jeewandara C, Ranasinghe P. Enhancing immunity in viral infections, with special emphasis on COVID-19: a review. *Diabetes Metab Syndr.* (2020) 14:367–82. doi: 10.1016/j.dsx.2020.04.015
122. Ilie PC, Stefanescu S, Smith L. The role of vitamin D in the prevention of coronavirus disease 2019 infection and mortality. *Aging Clin Exp Res.* (2020) 32:1195–8. doi: 10.1007/s40520-020-01570-8
123. Garami AR. Re: preventing a covid-19 pandemic - Is there a magic bullet to save COVID-19 patients? We can give it a try! *BMJ.* (2020) 368:m810. doi: 10.1136/bmj.m810
124. D'Avolio A, Avataneo V, Manca A, Cusato J, De Nicolò A, Lucchini R, et al. 25-hydroxyvitamin D concentrations are lower in patients with positive PCR for SARS-CoV-2. *Nutrients.* (2020) 12:1359. doi: 10.3390/nu12051359
125. Adams KK, Baker WL, Sobieraj DM. Myth busters: dietary supplements and COVID-19. *Ann Pharmacother.* (2020) 54:820–6. doi: 10.1177/1060028020928052

126. Mansur J. Letter: low population mortality from COVID-19 in countries south of latitude 35 degrees North supports vitamin D as a factor determining severity. *Aliment Pharmacol Ther.* (2020) 52:411–2. doi: 10.1111/apt.15820
127. Rhodes JM, Subramanian S, Laird E, Anne Kenny R. Letter: low population mortality from COVID-19 in countries south of latitude 35 degrees North supports vitamin D as a factor determining severity. Authors' reply. *Aliment Pharmacol Ther.* (2020) 52:412–3. doi: 10.1111/apt.15823
128. Zhang J, Xie B, Hashimoto K. Current status of potential therapeutic candidates for the COVID-19 crisis. *Brain Behav Immun.* (2020) 87:59–73. doi: 10.1016/j.bbi.2020.04.046
129. DeRobbio D. MUSC vitamin D research team to study connection to COVID-19 prevention and treatment. *WCIV* (2020). Available online at: https://abcnews4.com/news/coronavirus/musc-vitamin-d-research-team-to-study-connection-to-covid-19-prevention-and-treatment (accessed May 4, 2020).
130. Reporter S. Countries with Low Vitamin D Have Higher COVID-19 Infection and Mortality Rates: Here are They. *Science Times* (2020). Available online at: https://www.sciencetimes.com/articles/25567/20200504/coronavirus-countries-low-vitamin-d-high-risk-getting-disease.htm (accessed May 4, 2020).
131. Louisiana State University Health Sciences Center in New Orleans. *The LEAD COVID-19 Trial: Low-risk, Early Aspirin and Vitamin D to Reduce COVID-19 Hospitalizations*. clinicaltrials.gov (2020). Available online at: https://clinicaltrials.gov/ct2/show/NCT04363840 (accessed May 18, 2020).
132. Tameside General Hospital. *Investigating the Role of Vitamin D in the Morbidity of COVID-19 Patients*. clinicaltrials.gov (2020). Available online at: https://clinicaltrials.gov/ct2/show/NCT04386044 (accessed May 18, 2020).
133. VITACOV: *Vitamin D Polymorphisms and Severity of COVID-19 Infection - Full Text View - ClinicalTrials.gov*. Available online at: https://clinicaltrials.gov/ct2/show/NCT04370808 (accessed May 19, 2020).
134. Montano-Loza *A. Improving Vitamin D Status in the Management of COVID-19*. clinicaltrials.gov (2020). Available online at: https://clinicaltrials.gov/ct2/show/NCT04385940 (accessed May 18, 2020).
135. Garzón MC. *Effect of Vitamin D Administration on Prevention and Treatment of Mild Forms of Suspected Covid-19*. clinicaltrials.gov (2020). Available online at: https://clinicaltrials.gov/ct2/show/NCT04334005 (accessed May 18, 2020).
136. Dandinoglu T. *Do Vitamin D Levels Really Correlated With Disease Severity in COVID-19 Patients?* clinicaltrials.gov (2020). Available online at: https://clinicaltrials.gov/ct2/show/NCT04394390 (accessed May 18, 2020).
137. *COvid-19 and Vitamin D Supplementation: a Multicenter Randomized Controlled Trial of High Dose Versus Standard Dose Vitamin D3 in High-risk COVID-19 Patients (CoVitTrial) - Full Text View - ClinicalTrials.gov*. Available online at: https://clinicaltrials.gov/ct2/show/NCT04344041 (accessed May 19, 2020).
138. University Hospital, Lille. *Impact of Zinc and Vitamin D3 Supplementation on the Survival of Institutionalized Aged Patients Infected With COVID-19*. clinicaltrials.gov (2020). Available online at: https://clinicaltrials.gov/ct2/show/NCT04351490 (accessed May 18, 2020).
139. Sanford Health. *Randomized, Double-Blind, Controlled Trial of Hydroxychloroquine vs Placebo as Post-Exposure Prophylaxis Against COVID-19 Infection*. clinicaltrials.gov (2020). Available online at: https://clinicaltrials.gov/ct2/show/NCT04372017 (accessed May 18, 2020).
140. ProgenaBiome. *A Randomized, Double-Blind, Placebo-Controlled Phase IIa Study of Quintuple Therapy to Treat COVID-19 Infection*. clinicaltrials.gov (2020). Available online at: https://clinicaltrials.gov/ct2/show/NCT04334512 (accessed May 18, 2020).
141. *Oral 25-hydroxyvitamin D3 and COVID-19 - Full Text View - ClinicalTrials.gov*. Available online at: https://clinicaltrials.gov/ct2/show/NCT04386850 (accessed May 19, 2020).
142. dalili nooshin. *Effects of Standard Protocol Therapy With or Without Colchicine in Covid-19 Infection: A Randomized Double Blind Clinical Trial*. clinicaltrials.gov (2020). Available online at: https://clinicaltrials.gov/ct2/show/NCT04360980 (accessed May 18, 2020).
143. Ricordi C. *Umbilical Cord-Derived Mesenchymal Stem Cells for COVID-19 Patients With Acute Respiratory Distress Syndrome (ARDS)*. clinicaltrials.gov (2020). Available online at: https://clinicaltrials.gov/ct2/show/NCT04355728 (accessed May 18, 2020).
144. *Urinary Incontinence and Sedentary Behaviour in Nursing Homes - Full Text View - ClinicalTrials.gov*. Available online at: https://clinicaltrials.gov/ct2/show/NCT04297904 (accessed May 19, 2020).
145. *Prevention and Treatment With Calcifediol of COVID-19 Induced Acute Respiratory Syndrome - Full Text View - ClinicalTrials.gov*. Available online at: https://clinicaltrials.gov/ct2/show/NCT04366908 (accessed May 19, 2020).
146. Braiman M. *Latitude Dependence of the COVID-19 Mortality Rate—A Possible Relationship to Vitamin D Deficiency?* Rochester, NY: Social Science Research Network (2020). doi: 10.2139/ssrn.3561958
147. Gruber-Bzura BM. Vitamin D and influenza—prevention or therapy? *Int J Mol Sci.* (2018) 19:2419. doi: 10.3390/ijms19082419
148. Sabetta JR, DePetrillo P, Cipriani RJ, Smardin J, Burns LA, Landry ML. Serum 25-hydroxyvitamin D and the incidence of acute viral respiratory tract infections in healthy adults. *PLoS ONE.* (2010) 5:e11088. doi: 10.1371/journal.pone.0011088
149. Aranow C. Vitamin D and the immune system. *J Invest Med.* (2011) 59:881–6. doi: 10.2310/JIM.0b013e31821b8755
150. Bäcker A. *Slower COVID-19 Morbidity and Mortality Growth at Higher Solar Irradiance and Elevation*. Rochester, NY: Social Science Research Network (2020). doi: 10.2139/ssrn.3604729
151. Arboleda JF, Urcuqui-Inchima S. Vitamin D supplementation: a potential approach for COVID-19 therapeutics? *Front Immunol.* (2020) 11:1523. doi: 10.3389/fimmu.2020.01523
152. Ardiaria M. PERAN VITAMIN D DALAM PENCEGAHAN INFLUENZA DAN COVID-19. *J Nutr Health.* (2020) 8:79–85. doi: 10.14710/jnh.8.2.2020.79-85
153. Arya A, Dwivedi VD. Synergistic effect of Vitamin D and Remdesivir can fight COVID-19. *J Biomol Struct Dyn.* (2020) 1–2. doi: 10.1080/07391102.2020.1773929
154. Aslan MT, Aslan IÖ, Özdemir Ö. Is vitamin D one of the key elements in COVID-19 days? *J Nutr Health Aging.* (2020). doi: 10.1007/s12603-020-1413-5
155. Mohammed Hamad MN. Vitamin D supplements improve efficacy of minocycline, N-Acetylcysteine and aspirin triple therapy to COVID-19 infection. *SJBR.* (2020) 5:59–60. doi: 10.36348/sjbr.2020.v05i04.005
156. Hobday RA, Cason JW. The open-air treatment of PANDEMIC INFLUENZA. *Am J Public Health.* (2009) 99:S236–42. doi: 10.2105/AJPH.2008.134627
157. Lau FH, Majumder R, Torabi R, Saeg F, Hoffman R, Cirillo JD, et al. Vitamin D Insufficiency is Prevalent in Severe COVID-19. *medRxiv*. (2020) 2020.04.24.20075838. doi: 10.1101/2020.04.24.20075838
158. Tobolowsky FA. COVID-19 outbreak among three affiliated homeless service sites — King County, Washington, 2020. *MMWR Morb Mortal Wkly Rep.* (2020) 69:523–6. doi: 10.15585/mmwr.mm6917e2
159. Baggett TP, Keyes H, Sporn N, Gaeta JM. Prevalence of SARS-CoV-2 infection in residents of a large homeless shelter in Boston. *JAMA.* (2020) 323:2191–2. doi: 10.1001/jama.2020.6887
160. de Lucena TMC, da Silva Santos AF, de Lima BR, de Albuquerque Borborema ME, de Azevêdo Silva J. Mechanism of inflammatory response in associated comorbidities in COVID-19. *Diabetes Metab Syndr.* (2020) 14:597–600. doi: 10.1016/j.dsx.2020.05.025
161. Cantorna MT, Yu S, Bruce D. The paradoxical effects of vitamin D on Type 1 mediated immunity. *Mol Aspects Med.* (2008) 29:369–75. doi: 10.1016/j.mam.2008.04.004
162. Mohammad S, Mishra A, Ashraf MZ. Emerging role of vitamin D and its associated molecules in pathways related to pathogenesis of thrombosis. *Biomolecules.* (2019) 9:649. doi: 10.3390/biom9110649
163. Sundaram ME, Coleman LA. Vitamin D and influenza. *Adv Nutr.* (2012) 3:517–25. doi: 10.3945/an.112.002162
164. Cannell JJ, Vieth R, Umhau JC, Holick MF, Grant WB, Madronich S, et al. Epidemic influenza and vitamin D. *Epidemiol Infect.* (2006) 134:1129–40. doi: 10.1017/S0950268806007175
165. Martín Giménez VM, Inserra F, Tajer CD, Mariani J, Ferder L, Reiter RJ, et al. Lungs as target of COVID-19 infection: protective common

molecular mechanisms of vitamin D and melatonin as a new potential synergistic treatment. *Life Sci.* (2020) 254:117808. doi: 10.1016/j.lfs.2020.117808

166. Sparavigna, AC. Vitamin D for Covid-19? *Zenodo.* (2020). doi: 10.5281/ZENODO.3822187
167. Meftahi GH, Jangravi Z, Sahraei H, Bahari Z. The possible pathophysiology mechanism of cytokine storm in elderly adults with COVID-19 infection: the contribution of "inflame-aging." *Inflamm Res.* (2020) doi: 10.1007/s00011-020-01372-8
168. Biesalski HK. Vitamin D deficiency and co-morbidities in COVID-19 patients – A fatal relationship? *NFS J.* (2020) 20:10–21. doi: 10.1016/j.nfs.2020.06.001
169. Weir EK, Thenappan T, Bhargava M, Chen Y. Does vitamin D deficiency increase the severity of COVID-19? *Clin Med.* (2020) 1–2. doi: 10.7861/clinmed.2020-0301
170. Ulhaq ZS, Soraya GV. Interleukin-6 as a potential biomarker of COVID-19 progression. *Méd Maladies Infect.* (2020) 50:382–3. doi: 10.1016/j.medmal.2020.04.002
171. Silberstein M. Premorbid IL-6 levels may predict mortality from COVID-19. *Res Square.* (2020). doi: 10.21203/rs.3.rs-31814/v1
172. Froum S. Vitamin D and COVID-19 disease severity: An old ally in a new war. *Perio-Implant Advisory* (2020). Available online at: https://www.perioimplantadvisory.com/clinical-tips/article/14176684/vitamin-d-and-covid19-disease-severity-an-old-ally-in-a-new-war (accessed June 16, 2020).
173. Speeckaert MM, Delanghe JR. Association between low vitamin D and COVID-19: don't forget the vitamin D binding protein. *Aging Clin Exp Res.* (2020). doi: 10.1007/s40520-020-01607-y
174. Daneshkhah A, Agrawal V, Eshein A, Subramanian H, Roy HK, Backman V. The possible role of vitamin D in suppressing cytokine storm and associated mortality in COVID-19 Patients. *medRxiv.* (2020) 2020.04.08.20058578. doi: 10.1101/2020.04.08.20058578
175. Muscogiuri G, Pugliese G, Barrea L, Savastano S, Colao A. Obesity: the "Achilles heel" for COVID-19? *Metabolism.* (2020). doi: 10.1016/j.metabol.2020.154251. [Epub ahead of print].
176. Huibers MHW, Visser DH, Deckers MML, van Schoor NM, van Furth AM, Wolf BHM. Vitamin D deficiency among native Dutch and first- and second-generation non-Western immigrants. *Eur J Pediatr.* (2014) 173:583–8. doi: 10.1007/s00431-013-2198-x
177. Yousef S, Elliott J, Manuel D, Colman I, Papadimitropoulos M, Hossain A, et al. Study protocol: worldwide comparison of vitamin D status of immigrants from different ethnic origins and native-born populations—a systematic review and meta-analysis. *Syst Rev.* (2019) 8:211. doi: 10.1186/s13643-019-1123-4
178. Sääf M, Fernell E, Kristiansson F, Barnevik Olsson M, Gustafsson SA, et al.. Severe vitamin D deficiency in pregnant women of Somali origin living in Sweden: Vitamin D deficiency in pregnant Somali women. *Acta Paediatr.* (2011) 100:612–4. doi: 10.1111/j.1651-2227.2011.02134.x
179. Gasmi A, Noor S, Tippairote T, Dadar M, Menzel A, Bjørklund G. Individual risk management strategy and potential therapeutic options for the COVID-19 pandemic. *Clin Immunol.* (2020) 215:108409. doi: 10.1016/j.clim.2020.108409
180. Holick Michael F, Matsuoka Lois Y, Wortsman J. Age, vitamin D, and solar ultraviolet. *Lancet.* (1989) 334:1104–5. doi: 10.1016/S0140-6736(89)91124-0
181. Grant WB, Anouti FA, Moukayed M. Targeted 25-hydroxyvitamin D concentration measurements and vitamin D 3 supplementation can have important patient and public health benefits. *Eur J Clin Nutr.* (2020) 74:366–76. doi: 10.1038/s41430-020-0564-0
182. Bäcker A, Mageswaran M. *Double COVID-19 Confirmed Case Fatality Rate in Countries with High Elderly Female Vitamin D Deficiency Prevalence*. Rochester, NY: Social Science Research Network (2020). doi: 10.2139/ssrn.3623662
183. Annweiler C, Cao Z, Wu Y, Faucon E, Mouhat S, Kovacic H, et al. Counter-regulatory "Renin-angiotensin" system-based candidate drugs to treat COVID-19 diseases in SARS-CoV-2-infected patients. *Infect Disord Drug Targets.* (2020). doi: 10.2174/1871526520666200518073329. [Epub ahead of print].
184. Walker J, Hiramoto J, Gasper W, Auyang P, Conte MS, Rapp J, et al. Vitamin D deficiency is associated with mortality and adverse vascular access outcomes in patients with end stage renal disease. *J Vasc Surg.* (2014) 60:176–83. doi: 10.1016/j.jvs.2014.01.037
185. Kennel KA, Drake MT, Hurley DL. Vitamin D deficiency in adults: when to test and how to treat. *Mayo Clin Proc.* (2010) 85:752–8. doi: 10.4065/mcp.2010.0138
186. Lyons I, Ward V, Johnson J, Horton H. These are the health workers who have died from coronavirus. *The Telegraph* (2020). Available online at: https://www.telegraph.co.uk/news/0/nhs-workers-died-coronavirus-frontline-victims/ (accessed April 29, 2020).
187. Swerling G, Penna D. Why are so many black and ethnic minority people dying from coronavirus? *The Telegraph* (2020). Available online at: https://www.telegraph.co.uk/news/0/bame-coronavirus-black-ethnic-minority/ (accessed May 2, 2020).
188. Ahmed I, Azhar A, Eltaweel N, Tan BK. First Covid-19 maternal mortality in the UK associated with thrombotic complications. *Br J Haematol.* (2020) 190:e37–8. doi: 10.1111/bjh.16849
189. Muscogiuri G, Barrea L, Savastano S, Colao A. Nutritional recommendations for CoVID-19 quarantine. *Eur J Clin Nutr.* (2020) 74:850–1. doi: 10.1038/s41430-020-0635-2
190. Ekiz T, Pazarli AC. Relationship between COVID-19 and obesity. *Diabetes Metab Syndr Clin Res Rev.* (2020) 14:761–3. doi: 10.1016/j.dsx.2020.05.047
191. Palaniveloo L, Yong HY, Mohd Shariff Z, Peng LS, Bindels J, Tee YYS, et al. Vitamin D status is associated with high BMI, working status and gravidity among pregnant Malaysian women. *Mal J Nutr.* (2020) 26:129–39. doi: 10.31246/mjn-2020-0010
192. Ekiz T, Kara M, Özçakar L. Revisiting vitamin D and home-based exercises for patients with sleep apnea facing the COVID-19 quarantine. *J Clin Sleep Med.* (2020). doi: 10.5664/jcsm.8586
193. Khunti K, Singh AK, Pareek M, Hanif W. Is ethnicity linked to incidence or outcomes of covid-19? *BMJ.* (2020) 369:m1548. doi: 10.1136/bmj.m1548
194. Facchiano A, Facchiano A, Bartoli M, Ricci A, Facchiano F. Reply to Jakovac: about COVID-19 and vitamin D. *Am J Physiol Endocrinol Metab.* (2020) 318:E838. doi: 10.1152/ajpendo.00185.2020
195. Yao Y, Pan J, Liu Z, Meng X, Wang W, Kan H, Wang W. No Association of COVID-19 transmission with temperature or UV radiation in Chinese cities. *Eur Respir J.* (2020) 55:2000517. doi: 10.1183/13993003.00517-2020
196. Chinazzi M, Davis JT, Ajelli M, Gioannini C, Litvinova M, Merler S, et al. The effect of travel restrictions on the spread of the 2019 novel coronavirus (COVID-19) outbreak. *Science.* (2020) 368:395–400. doi: 10.1126/science.aba9757
197. Li AY, Hannah TC, Durbin JR, Dreher N, McAuley FM, Marayati NF, et al. Multivariate analysis of black race and environmental temperature on COVID-19 in the US. *Am J Med Sci.* (2020). doi: 10.1016/j.amjms.2020.06.015
198. Panarese A, Shahini E. Letter: Covid-19, and vitamin D. *Aliment Pharmacol Ther.* (2020) 51:993–5. doi: 10.1111/apt.15752
199. Kumar V, Srivastava A. Spurious Correlation? A review of the relationship between Vitamin D and Covid-19 infection and mortality. *medRxiv.* (2020) 2020.05.25.20110338. doi: 10.1101/2020.05.25.20110338 (Preprint)
200. Singh S, Kaur R, Singh RK. Revisiting the role of vitamin D levels in the prevention of COVID-19 infection and mortality in European countries post infections peak. *Res Square.* (2020) doi: 10.21203/rs.3.rs-32484/v1
201. Notari A, Torrieri G. COVID-19 transmission risk factors. *arXiv preprint* arXiv:200503651 (2020).
202. *Coronavirus Update (Live): 4,005,655 Cases and 275,669 Deaths from COVID-19 Virus Pandemic - Worldometer*. Available online at: https://www.worldometers.info/coronavirus/#countries (accessed May 8, 2020).
203. Osmancevic A, Demeke T, Gillstedt M, Angesjö E, Sinclair H, Abd El-Gawad G, et al. Vitamin D treatment in Somali women living in Sweden - Two randomized, placebo-controlled studies. *Clin Endocrinol.* (2016) 85:535–43. doi: 10.1111/cen.13097
204. Bäcker A. *Non-black Hispanic/Latinos Spend More Time Outdoors and Have Lower COVID-19 Morbidity Than Non-Hispanic/Latinos*. Rochester, NY: Social Science Research Network (2020). doi: 10.2139/ssrn.3575217
205. Li M, Zhang Z, Cao W, Liu Y, Du B, Chen C, et al. Identifying novel factors associated with COVID-19 transmission and

fatality using the machine learning approach. *medRxiv*. (2020) 2020.06.10.20127472. doi: 10.1101/2020.06.10.20127472
206. Kohlmeier M. Avoidance of vitamin D deficiency to slow the COVID-19 pandemic. *BMJ Nutr Prev Health*. (2020) bmjnph-2020-000096. doi: 10.1136/bmjnph-2020-000096
207. Alipio MM. Do latitude and ozone concentration predict Covid-2019 cases in 34 countries? *medRxiv*. (2020) 2020.04.09.20060202. doi: 10.1101/2020.04.09.20060202
208. Skutsch M, Dobler C, McCall MBB, Ghilardi A, Salinas-Melgoza MA, McCall MK, et al. The association of UV with rates of COVID-19 transmission and deaths in Mexico: the possible mediating role of vitamin D. *medRxiv*. (2020) 2020.05.25.20112805. doi: 10.1101/2020.05.25.20112805
209. Li Y, Li Q, Zhang N, Liu Z. Sunlight and vitamin D in the prevention of coronavirus disease (COVID-19) infection and mortality in the United States. *Res Square* (2020). doi: 10.21203/rs.3.rs-32499/v1
210. Marik PE, Kory P, Varon J. Does vitamin D status impact mortality from SARS-CoV-2 infection? *Med Drug Discov*. (2020) 6:100041. doi: 10.1016/j.medidd.2020.100041
211. Annweiler C, Cao Z, Sabatier J-M. Point of view: should COVID-19 patients be supplemented with vitamin D? *Maturitas*. (2020) 140:24–6. doi: 10.1016/j.maturitas.2020.06.003
212. Horowitz RI, Freeman PR, Bruzzese J. Efficacy of glutathione therapy in relieving dyspnea associated with COVID-19 pneumonia: a report of 2 cases. *Respir Med Case Rep*. (2020) 30:101063. doi: 10.1016/j.rmcr.2020.101063
213. Bossoni S, Chiesa L, Giustina A. Severe hypocalcemia in a thyroidectomized woman with Covid-19 infection. *Endocrine*. (2020). doi: 10.1007/s12020-020-02326-0. [Epub ahead of print].
214. Pinzon RT, Angela A, Pradana AW. Vitamin d deficiency among patients with COVID-19 : case series and recent literature review. *Res Square*. (2020). doi: 10.21203/rs.3.rs-29473/v1
215. Faul JL, Kerley C, Love B, O-Nell E, Cody C, Tormey W, et al. Vitamin D deficiency and ARDS after SARS-CoV-2 infection. *Ir Med J*. (2020) 113:84.
216. Fasano A, Cereda E, Barichella M, Cassani E, Ferri V, Zecchinelli AL, et al. COVID-19 in Parkinson's disease patients living in Lombardy, Italy. *Mov Disord*. (2020) 35:1089–93. doi: 10.1002/mds.28176
217. Tan CW, Ho LP, Kalimuddin S, Cherng BPZ, Teh YE, Thien SY, et al. A cohort study to evaluate the effect of combination Vitamin D, Magnesium and Vitamin B12 (DMB) on progression to severe outcome in older COVID-19 patients. *medRxiv*. (2020) 2020.06.01.20112334. doi: 10.1101/2020.06.01.20112334
218. Asyary A, Veruswati M. Sunlight exposure increased Covid-19 recovery rates: a study in the central pandemic area of Indonesia. *Sci Total Environ*. (2020). doi: 10.1016/j.scitotenv.2020.139016
219. Sun J-K, Zhang W-H, Zou L, Liu Y, Li J-J, Kan X-H, et al. Serum calcium as a biomarker of clinical severity and prognosis in patients with coronavirus disease 2019: a retrospective cross-sectional study. *Res Square*. (2020). doi: 10.21203/rs.3.rs-17575/v1
220. Cuñat T, Ojeda A, Calvo A. Vitamin D deficiency in critically ill patients diagnosed with COVID−19. Are we doing enough? A retrospective analysis of 226 patients. *Res Square*. (2020). doi: 10.21203/rs.3.rs-30390/v1
221. De Smet D, De Smet K, Herroelen P, Gryspeerdt S, Martens GA. Vitamin D deficiency as risk factor for severe COVID-19: a convergence of two pandemics. *medRxiv*. (2020) 2020.05.01.20079376. doi: 10.1101/2020.05.01.20079376
222. Meltzer DO, Best TJ, Zhang H, Vokes T, Arora V, Solway J. Association of Vitamin D deficiency and treatment with COVID-19 incidence. *medRxiv*. (2020) 2020.05.08.20095893. doi: 10.1101/2020.05.08.20095893
223. Matos RI, Chung KK. *DoD COVID-19 Practice Management Guide Clinical Management of COVID-19 | Technical Resources | ASPR TRACIE*. (2020). Available online at: https://asprtracie.hhs.gov/technical-resources/resource/7899/dod-covid-19-practice-management-guide-clinical-management-of-covid-19 (accessed May 20, 2020).
224. Dasgupta J, Sen U, Bakshi A, Dasgupta A, Manna K, Saha C, et al. Nsp7 and spike glycoprotein of SARS-CoV-2 Are Envisaged as potential targets of vitamin D and ivermectin. *Preprints*. (2020). doi: 10.20944/preprints202005.0084.v1
225. Lanham-New SA, Webb AR, Cashman KD, Buttriss JL, Fallowfield JL, Masud T, et al. Vitamin D and SARS-CoV-2 virus/COVID-19 disease. *BMJ Nutr Prev Health*. (2020) bmjnph-2020-000089. doi: 10.1136/bmjnph-2020-000089
226. Pal R, Bhadada SK. Managing common endocrine disorders amid COVID-19 pandemic. *Diabetes Metab Syndr Clin Res Rev*. (2020) 14:767–71. doi: 10.1016/j.dsx.2020.05.050
227. Nizami NS, Mujeebuddin CS. Strong Immunity - A Major Weapon to Fight against Covid-19. *IOSR J Pharm Biol Sci*. (2020) 15:22–9. doi: 10.9790/1503032229
228. Richards G, Mer M, Schleicher G, Stacey S. COVID-19 and the rationale for pharmacotherapy : a South African perspective. *Wits J Clin Med*. (2020) 2:11–8. doi: 10.18772/26180197.2020.v2nSIa2
229. Wimalawansa SJ. Global epidemic of coronavirus—Covid-19: what can we do to minimize risks. *Eur J Biomed Pharm Sci*. (2020) 7:7. Available online at: http://www.ejbps.com
230. Puig-Domingo M, Marazuela M, Giustina A. COVID-19 and endocrine diseases. A statement from the European Society of Endocrinology. *Endocrine*. (2020) 68:2–5. doi: 10.1007/s12020-020-02294-5
231. Uçar D, Tayfun K, Müslümanoglu AY, Kalayci MZ. Coronavirus ve Fitoterapi. *Bütünleyici Anadolu Tibbi Dergisi* (2020) 1:49–57.
232. Ziccarelli V. Nutrition therapy for severe viral infections (COVID-19): recommendations and considerations for integrative medical treatments. *J Orthomol Med*. (2020) 35.
233. Koenig HG. Ways of protecting religious older adults from the consequences of COVID-19. *Am J Geriatr Psychiatry*. (2020) 28:776–9. doi: 10.1016/j.jagp.2020.04.004
234. Suresh PS. Hypovitaminosis D and COVID-19: matter of concern in India? *Ind J Clin Biochem*. (2020) 35:378–9. doi: 10.1007/s12291-020-00894-6
235. Queiroz NSF, Barros LL, de Azevedo MFC, Oba J, Sobrado CW, de Sousa Carlos A, et al. Management of inflammatory bowel disease patients in the COVID-19 pandemic era: a Brazilian tertiary referral center guidance. *Clinics*. (2020) 75:e1909. doi: 10.6061/clinics/2020/e1909
236. Nikhra V. Exploring pathophysiology of COVID-19 infection: faux espoir and dormant therapeutic options. *Int J Clin Virol*. (2020) 4:56–64. doi: 10.29328/journal.ijcv.1001016
237. Docea AO, Tsatsakis A, Albulescu D, Cristea O, Zlatian O, Vinceti M, et al. A new threat from an old enemy: re-emergence of coronavirus (Review). *Int J Mol Med*. (2020) 45:1631–43. doi: 10.3892/ijmm.2020.4555
238. Gee S, Gaughran F, MacCabe J, Shergill S, Whiskey E, Taylor D. Management of clozapine treatment during the COVID-19 pandemic. *Ther Adv Psychopharmacol*. (2020). doi: 10.1177/2045125320928167
239. Kurthkoti K, Das G. Potential benefits of Vitamin D supplementation in COVID-19 patients: a hypothesis. *OSF Preprints*. (2020). doi: 10.31219/osf.io/dnjav
240. Ramos EM, de Abreu AC, de Freitas SLF, de Lima MD, dos Reis FJM, Ramos HV, et al. COVID-19, rate of case factors and nutritional characteristics of patients dying in Italy and Brazil: a critical analyze. *Global J Health Sci*. (2020) 12:133. doi: 10.5539/gjhs.v12n7p133
241. Razdan K, Singh K, Singh D. Vitamin D levels and COVID-19 susceptibility: is there any correlation? *Med Drug Discov*. (2020) 7:100051. doi: 10.1016/j.medidd.2020.100051
242. Saul AW. Nutritional Treatment of Coronavirus. *Orthomolecular Medicine News Service* (2020). Available online at: http://orthomolecular.org/resources/omns/v16n06.shtml
243. Siuka D, Pfeifer M, Pinter B. Vitamin D supplementation in the COVID-19 pandemic. *Mayo Clin Proc*. (2020). doi: 10.1016/j.mayocp.2020.05.036
244. Tan SHS, Hong CC, Saha S, Murphy D, Hui JH. Medications in COVID-19 patients: summarizing the current literature from an orthopaedic perspective. *Int Orthop*. (2020) 44:1599–603. doi: 10.1007/s00264-020-04643-5
245. Tian Y, Rong L. Letter: does vitamin D have a potential role against COVID-19? Authors' reply. *Aliment Pharmacol Ther*. (2020) 52:410–1. doi: 10.1111/apt.15817
246. Yousfi N, Bragazzi NL, Briki W, Zmijewski P, Chamari K. The COVID-19 pandemic: how to maintain a healthy immune system during the quarantine - a multidisciplinary approach with special focus on athletes. *Biol Sport*. (2020) 37:211–6. doi: 10.5114/biolsport.2020.95125

247. Zabetakis I, Lordan R, Norton C, Tsoupras A. COVID-19: the inflammation link and the role of nutrition in potential mitigation. *Nutrients.* (2020) 12:1466. doi: 10.3390/nu12051466
248. Zemb P, Bergman P, Camargo CA, Cavalier E, Cormier C, Courbebaisse M, et al. Vitamin D deficiency and COVID-19 pandemic. *J Glob Antimicrob Resist.* (2020) 22:133–4. doi: 10.1016/j.jgar.2020.05.006
249. Piller C. 'This is insane!' Many scientists lament Trump's embrace of risky malaria drugs for coronavirus. *Science | AAAS* (2020). Available online at: https://www.sciencemag.org/news/2020/03/insane-many-scientists-lament-trump-s-embrace-risky-malaria-drugs-coronavirus (accessed April 23, 2020).
250. NIH. *Office of Dietary Supplements - Vitamin D*. Available online at: https://ods.od.nih.gov/factsheets/VitaminD-HealthProfessional/ (accessed April 23, 2020).
251. McCartney DM, Byrne DG. McCartney et al. comment on 'Vitamin D and Covid-19 – A Note of Caution' response letter. *Ir Med J.* 113:79.
252. Cronise RJ, Sinclair DA, Bremer AA. The "metabolic winter" hypothesis: a cause of the current epidemics of obesity and cardiometabolic disease. *Metab Syndr Relat Disord.* (2014) 12:355–61. doi: 10.1089/met.2014.0027
253. Siddiqui R, Ahmed Khan N. Centralized air-conditioning and transmission of novel coronavirus. *Pathog Glob Health.* (2020) 1–2. doi: 10.1080/20477724.2020.1765653
254. Nigro E, Polito R, Alfieri A, Mancini A, Imperlini E, Elce A, et al. Molecular mechanisms involved in the positive effects of physical activity on coping with COVID-19 *Preprints.* (2020). 2020060084.

Conflict of Interest: LB is employed by Ferris Mfg. Corp, makers of PolyMem dressings, and declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 11 September 2020
doi: 10.3389/fimmu.2020.552909

Comparative Review of SARS-CoV-2, SARS-CoV, MERS-CoV, and Influenza A Respiratory Viruses

Zeinab Abdelrahman[1,2], *Mengyuan Li*[1,2] *and Xiaosheng Wang*[1,2]*

[1] Biomedical Informatics Research Lab, School of Basic Medicine and Clinical Pharmacy, China Pharmaceutical University, Nanjing, China, [2] Cancer Genomics Research Center, School of Basic Medicine and Clinical Pharmacy, China Pharmaceutical University, Nanjing, China

The 2019 novel coronavirus (SARS-CoV-2) pandemic has caused a global health emergency. The outbreak of this virus has raised a number of questions: What is SARS-CoV-2? How transmissible is SARS-CoV-2? How severely affected are patients infected with SARS-CoV-2? What are the risk factors for viral infection? What are the differences between this novel coronavirus and other coronaviruses? To answer these questions, we performed a comparative study of four pathogenic viruses that primarily attack the respiratory system and may cause death, namely, SARS-CoV-2, severe acute respiratory syndrome (SARS-CoV), Middle East respiratory syndrome (MERS-CoV), and influenza A viruses (H1N1 and H3N2 strains). This comparative study provides a critical evaluation of the origin, genomic features, transmission, and pathogenicity of these viruses. Because the coronavirus disease 2019 (COVID-19) pandemic caused by SARS-CoV-2 is ongoing, this evaluation may inform public health administrators and medical experts to aid in curbing the pandemic's progression.

Keywords: SARS-CoV-2, SARS-CoV, MERS-CoV, influenza A virus, COVID-19

OPEN ACCESS

Edited by:
Alexander Rodriguez-Palacios, Case Western Reserve University, United States

Reviewed by:
Shuvojit Banerjee, Case Western Reserve University, United States
Rachel Graham, The University of North Carolina at Chapel Hill, United States

***Correspondence:**
Xiaosheng Wang
xiaosheng.wang@cpu.edu.cn

Specialty section:
This article was submitted to Viral Immunology, a section of the journal Frontiers in Immunology

Received: *17 April 2020*
Accepted: *24 August 2020*
Published: *11 September 2020*

Citation:
Abdelrahman Z, Li M and Wang X (2020) Comparative Review of SARS-CoV-2, SARS-CoV, MERS-CoV, and Influenza A Respiratory Viruses. Front. Immunol. 11:552909. doi: 10.3389/fimmu.2020.552909

INTRODUCTION

The 2019 novel coronavirus (SARS-CoV-2), severe acute respiratory syndrome coronavirus (SARS-CoV), Middle East respiratory syndrome coronavirus (MERS-CoV), and influenza A viruses are major pathogens that primarily target the human respiratory system. Diseases associated with their infections vary from mild respiratory illness to acute pneumonia and even respiratory failure. Since 1918, the influenza A viruses have caused four pandemics. The first and most severe pandemic in recent history, known as "Spanish influenza," occurred in 1918 and was caused by an H1N1 influenza A virus (IAV) strain (1). Approximately 500 million people were infected, and 50 million people died during this pandemic. The second pandemic, known as "Asian influenza," occurred in 1957, was caused by an H2N2 IAV strain, and resulted in ∼1.1 million deaths worldwide (2). The third pandemic, known as "Hong Kong flu," occurred in 1968 and was caused by an H3N2 IAV strain, resulting in ∼1 million deaths worldwide (3). The fourth pandemic was caused by the influenza A (H1N1) pdm09 virus, also known as the "novel influenza A virus," and resulted in 151,700–575,400 deaths worldwide from 2009 to 2010 (4, 5). Since that time, the novel influenza A virus has continued to spread as a seasonal flu virus. From September 2019 to February 2020, this virus caused at least 34 million flu illnesses and 20,000 deaths. In November 2002, before the fourth influenza A pandemic, an epidemic caused by a betacoronavirus (SARS-CoV) and known as severe acute respiratory syndrome (SARS) began in South China and spread to 29 countries. The SARS outbreak caused ∼8,000 infections and 774 deaths before it was contained in July 2003, with

a case fatality rate (CFR) of 9.6% (the CFR was ∼50% among patients 65 or older) (6). However, since 2004, there have not been any SARS cases reported anywhere in the world. In September 2012, Saudi Arabia reported the first case of Middle East respiratory syndrome (MERS), which was caused by another type of betacoronavirus (MERS-CoV). MERS-CoV spread to 27 countries and caused 2,519 infections and 866 deaths by January 2020, with a CFR of 34.4% (7).

In December 2019, cases of the new coronavirus disease 2019 (COVID-19), caused by a new betacoronavirus (SARS-CoV-2), were first reported in Wuhan, China (8). These cases were characterized by acute pneumonia-associated symptoms, such as fever, dry cough, chills, shortness of breath, and muscle pain (9). The SARS-CoV-2 outbreak rapidly spread worldwide. It has infected more than 14 million individuals and resulted in more than 500,000 deaths as of 20 July 2020. In comparison with the other two coronaviruses, SARS-CoV-2 appears to be much more contagious and infectious; it has rapidly resulted in a pandemic constituting a global health emergency (**Figures 1A–C**).

To better understand the current COVID-19 pandemic caused by SARS-CoV-2, we have performed a comparative study between SARS-CoV-2 and past epidemic/pandemic viral infections that primarily affect the respiratory system: the influenza A viruses (H3N2 and H1N1 strains) and the two coronaviruses SARS-CoV and MERS-CoV. We have explored the genomic characteristics, transmission, reservoirs, and pathogenesis of these four pathogens. We have also considered the preventive and control measures conducted by the World Health Organization (WHO) against the spread of these pathogens. Additionally, we have elucidated how these viruses attack the immune system and the associated host immune system response. This comparative study will aid in informing public health administrators and medical experts on how to adequately distinguish between these viruses and identify the preventive and control measures recommended by the WHO against the spread of SARS-CoV-2.

A brief comparison between the four pathogenic viruses, including their characteristics, pathogenesis, and transmission, is summarized in **Table 1**.

TAXONOMY, STRUCTURE, AND GENOMIC PROPERTIES OF THE VIRUSES

Influenza A

Influenza A viruses that infect humans mainly consist of two strains (H1N1 and H3N2). Both strains are characterized as enveloped, negative-sense, single-stranded RNA viruses with a total genome size of ∼13.5 kb (18, 19). The influenza A virus genome consists of eight different segments, with each segment containing a region that encodes one or two proteins with specific functions, including hemagglutinin (HA), polymerase basic protein 2 (PB2), nucleoprotein (NP), polymerase basic protein 1 (PB1), neuraminidase (NA), matrix (M), nonstructural protein (NS1), and polymerase acidic protein (PA) (20, 21).

The HA protein of influenza A viruses binds to the glycoprotein terminal sialic acid and glycolipid receptors, which contain α-2,6 and α-2,3 sialic acid groups attached to galactose. Although HA is considered to be a more crucial antigenic determinant than NA, both proteins are potentially restrictive factors for viral evolution (20, 22). In addition, there are three viral polymerase proteins, PB1, PB2, and PA, encoded on segments 1, 2, and 3, respectively; these polymerase proteins form an enzyme complex that plays a role in transcription and replication. Finally, the NP protein encoded on segment 5 is used as a model to generate additional copies (23, 24).

Influenza A viruses exhibit antigenic drift/shift properties, allowing them to avoid the host immune response. The Centers for Disease Control and Prevention (CDC) defines antigenic drift as genetic variation that occurs in antigen structures owing to point mutations in the HA and NA genes over time, whereas antigenic shift is the result of a sudden genetic reassortment between two or more closely related influenza viral strains (23, 24). A well-known example of the antigenic shift phenomenon is the triple reassortment that occurred in the influenza A pdm09 virus and caused the 2009 pandemic as a result of the replacement of the hemagglutinin H2 and polymerase PB1 genes of the avian H2N2 virus with two new avian H3 and PB1 genes (25, 26) (**Figure 2A**). These antigenic drift/shift properties can potentially reduce the effectiveness of vaccines and become a considerable challenge in antiviral therapy (27, 28).

SARS-CoV

The coronavirus family is so named because of the large spike protein molecules that are present on the virus surface and gives the virions a crown-like shape; coronavirus genomes are the largest among RNA viruses (29). This family has been classified into at least three primary genera (alpha, beta, and gamma). Within this family, seven viruses are currently known to infect humans, namely, NL63 and 229E from the alpha genus and OC43, HKU1, SARS-CoV, MERS-CoV, and SARS-CoV-2 from the beta genus. SARS-CoV is a positive-stranded RNA virus belonging to the family *Coronaviridae* (30), order *Nidovirales*, genus *Betacoronavirus*, lineage B (from the International Committee on Taxonomy of Viruses). It was characterized as a giant, enveloped, positive-stranded RNA virus with a genome comprising 29,727 nucleotides (∼30 kb), 41% of which are guanine or cytosine. The genomic body of this virus has the original gene order of 5'-replicase (rep), which makes up approximately two-thirds of the genome and consists of the large genes ORF1a and ORF1b. ORF1a and ORF1b of the rep gene encode two large polyproteins known as pp1a (486 kDa) and pp1ab (790 kDa). In addition, the 3' structural spike (S), envelope (E), membrane (M), and nucleocapsid (N) proteins are encoded by four open reading frames (ORFs) downstream of the rep gene (31). The rep gene products are translated from genomic RNA, whereas the remaining viral proteins are translated from subgenomic mRNAs. In addition to the original genes, the SARS-CoV genome encodes another eight putative accessory proteins,

Abbreviations: SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SARS-CoV, severe acute respiratory syndrome coronavirus; MERS-CoV, the Middle East respiratory syndrome coronavirus; WHO, world health organization; CDC, center of disease control and prevention; nt, nucleotide; kb, kilobase; KDa, kilodalton molecular weight unit.

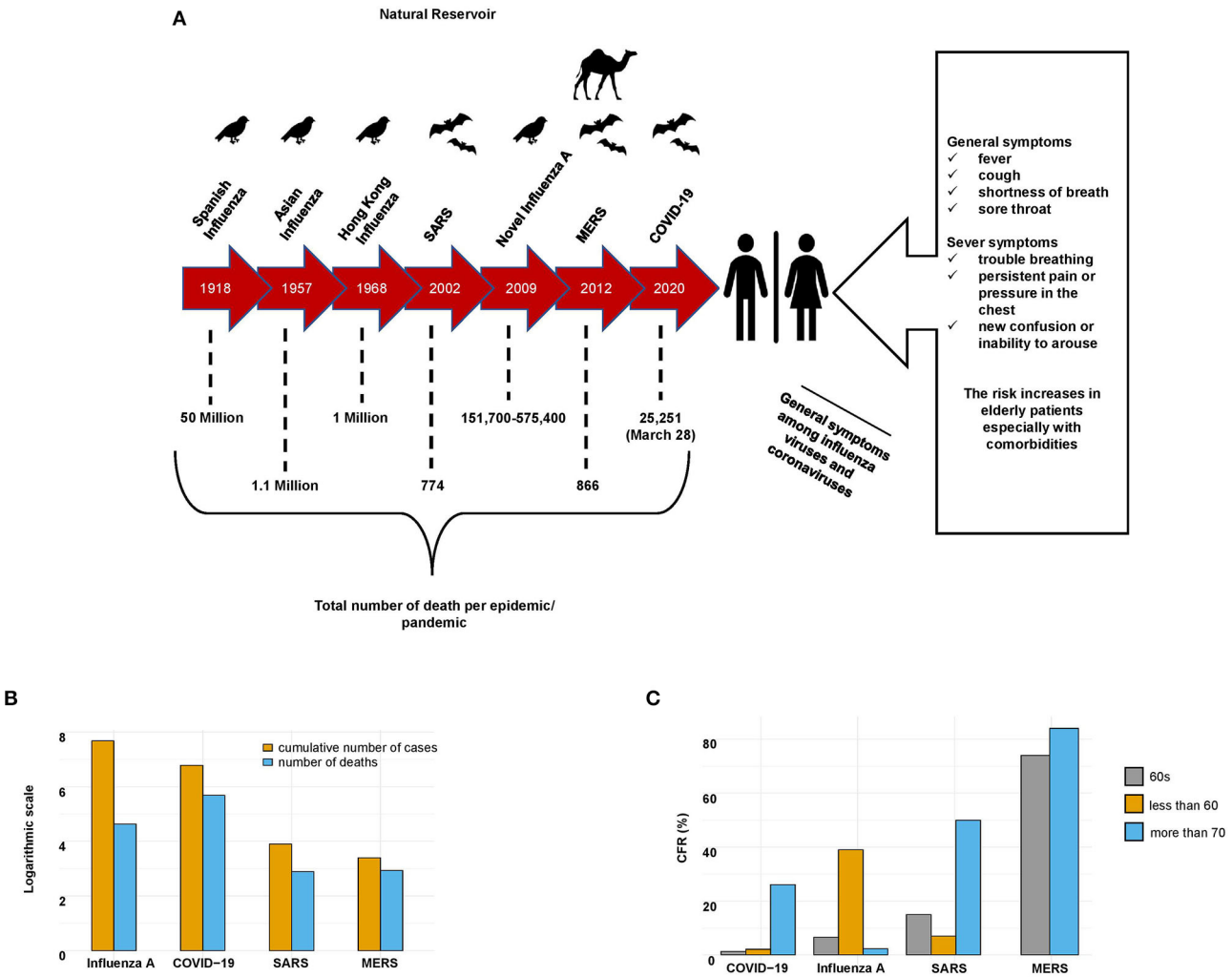


FIGURE 1 | General characteristics of SARS-CoV-2, SARS-CoV, MERS-CoV, and influenza A viruses. **(A)** Epidemics of SARS-CoV-2, SARS-CoV, MERS-CoV, and influenza A viruses. The timeline, natural reservoirs, total number of deaths, and symptoms of the patients infected with these viruses. **(B)** Cumulative numbers of cases and deaths caused by SARS-CoV-2, SARS-CoV, MERS-CoV, and influenza A (during the last seasonal flu 2019–2020) viruses. Influenza A virus infected the most people, while SARS-CoV-2 caused the most deaths. **(C)** Case-fatality rate (CFR) of patients infected with SARS-CoV-2, SARS-CoV, MERS-CoV, and influenza A (the last seasonal flu 2019–2020) viruses stratified by age.

known as ORFs 3a, 3b, 6, 7a, 7b, 8a, 8b, and 9b, which vary in length from 39 to 274 amino acids. Although the SARS-CoV rep gene and structural proteins have some sequence homology with other coronaviruses, the accessory proteins do not show substantial homology to the viral proteins of other coronaviruses at the amino acid level (31).

MERS-CoV

Although MERS-CoV belongs to the same family, order, and genus as SARS-CoV, it was the first betacoronavirus lineage C member identified as a "novel coronavirus" with a genome size of 30,119 nucleotides. The genome of MERS-CoV encodes 10 proteins. These 10 proteins comprise two replicase polyproteins (ORF1ab and ORF1a), four structural proteins (E, N, S, and M), and four nonstructural proteins (ORFs 3, 4a, 4b, and 5) (32). In addition to the rep and structural genes, there are accessory protein genes interspersed between the structural protein genes that may interfere with the host innate immune response in infected animals (7).

SARS-CoV-2

Although SARS-CoV-2 belongs to the same family and genus as SARS-CoV and MERS-CoV, genomic analysis revealed greater similarity between SARS-CoV-2 and SARS-CoV. Thus, researchers classified it as a member of lineage B (from the International Committee on Taxonomy of Viruses). Initially, the *Coronaviridae* Study Group of the International Committee on Taxonomy of Viruses identified this virus as a sister clade to the prototype human and bat severe acute respiratory syndrome coronaviruses (SARS-CoVs) of the species *Severe acute*

TABLE 1 | General characteristics of SARS-CoV-2, SARS-CoV, MERS-CoV, and influenza A viruses.

Characteristic	SARS-CoV-2	SARS-CoV	MERS-CoV	Influenza A
Year of the first reported case	2019	2002	2012	1918
Country/Region of the first reported case	China	China	Middle East	United States
Natural reservoir	Unclear (possibly bats)	Chinese horseshoe bats	Camels (possibly bats)	Birds
Intermediate host	Debatable (possibly pangolins) (10)	Civet cats	Dromedary camels	Pigs
Primary modes of transmission	Droplet, aerosol, and contact	Droplet, aerosol, and contact	Droplet, aerosol, and contact	Droplet, aerosol, and contact
Incubation period	2–14 days	2–7 days	2–14 days	2 days
Reproduction number (R_0)	$R_0 = 3.1$ (coefficient of determination, $r^2 = 0.99$)	Median: 0.58; IQR: 0.24–1.18	Mean: 0.69 (95% CI 0.50–0.92)	Median: 1.27; IQR: 1.19–1.37
Host receptor	ACE2	ACE2	DPP4	Sialic acid-containing molecules
Dominant cell entry pathway	Unclear	Clathrin- and caveolae-independent endocytic pathway (11)	Cell membrane fusion (12)	Receptor-mediated endocytosis (13)
Blood test results	Lymphopenia, thrombocytopenia, leukopenia, leucocytosis, monocytosis, and low CRP (14)	Lymphopenia, thrombocytopenia, and leukopenia (15)	Leucocytosis, monocytosis, and low CRP (16)	Lymphopenia, eosinopenia, hypoferremia, decreased levels of serum CO_2-CP, increased levels of serum CRP and serum CH50 (17)
Case fatality rate	1–3%	∼15%	34.4%	0.1%

IQR, interquartile range; CRP, C-reactive protein; CI, confidence interval; ACE2, Angiotensin-converting Enzyme 2; DPP4, Dipeptidyl peptidase-4 inhibitor; CO2-CP, carbon dioxide; CH50, Total Complement Activity.

respiratory syndrome-related coronavirus. Later, it was labeled as SARS-CoV-2 (33). The RNA genome size of SARS-CoV-2 is 30,000 bases in length. Among other betacoronaviruses, this virus is characterized by a unique combination of polybasic cleavage sites, a distinctive feature known to increase pathogenicity and transmissibility in other viruses (34).

Genomic analysis of SARS-CoV-2 revealed that the genome consists of six major ORFs and shares less than an 80% nucleotide sequence identity with SARS-CoV. However, the seven conserved replicase domains in the ORF1ab amino acid sequence share a 94.4% identity with those in SARS-CoV (35). Genomic analysis also revealed that the SARS-CoV-2 genome is highly similar to that of the bat coronavirus (Bat CoV RaTG13), with a sequence identity of 96.2%. Furthermore, the receptor-binding spike protein shares a 93.1% similarity to Bat CoV RaTG13 (35). Meanwhile, relative to SARS-CoV, significant differences were observed in the sequence of the S gene of SARS-CoV-2, including three short insertions in the N-terminal domain, changes in four out of five of the crucial residues in the receptor-binding motif, and the presence of an unexpected furin cleavage site at the S1/S2 boundary of the SARS-CoV-2 spike glycoprotein. This insertion is a novel feature that differentiates SARS-CoV-2 from SARS-CoV and several SARS-related coronaviruses (SARSr-CoVs) (36).

VIRAL ORIGIN AND EVOLUTION

Influenza A

Influenza A H1N1 and H3N2 subtype viruses are two of the three combinations known to have circulated widely in humans and to currently cause seasonal influenza; these strains originated from birds and swine. Before 1979, the only lineage detected in swine herds from Europe was the classical swine influenza virus A H1N1 lineage 1A (25). This strain shares a mutual ancestor with the virus that caused the 1918 human influenza A pandemic. However, in the early 1980s, the classical swine H1N1 strain was displaced by a new European enzootic swine influenza A viral strain: the Eurasian, avian-like H1N1 ($H1_{av}N1$) lineage 1C (26). After its rapid transmission from birds to mammals, the $H1_{av}N1$ virus underwent rapid and sustained adaptation in mammals. Furthermore, this virus has also undergone rapid reassortment, resulting in the appearance of multiple genotypes. The two primary enzootic subtypes are H1N2 (H1huN2) lineage IB and H3N2, which occurred through the acquisition of HA or NA gene segments originating from seasonal human influenza viruses (**Figure 2B**) (37).

As previously mentioned, influenza A exhibits antigenic drift/shift phenomena resulting from the HA protein's ability to undergo rapid evolution because of the plasticity of the viral RNA-dependent RNA polymerase. It is believed that mutations occurring in the HA protein, including reassortments and mutations among animals and humans, were the drivers of previous pandemics (38).

Adaptive mutations can lead to a number of phenotypic changes, including variations in antigenicity, increased diversity in viral protein sequences, the ability to avoid antibody pressure, receptor preference, virulence, altered fusion functionality, and evasion of the immune response. Rapid modifications can give rise to new strains with features that are different from any viruses

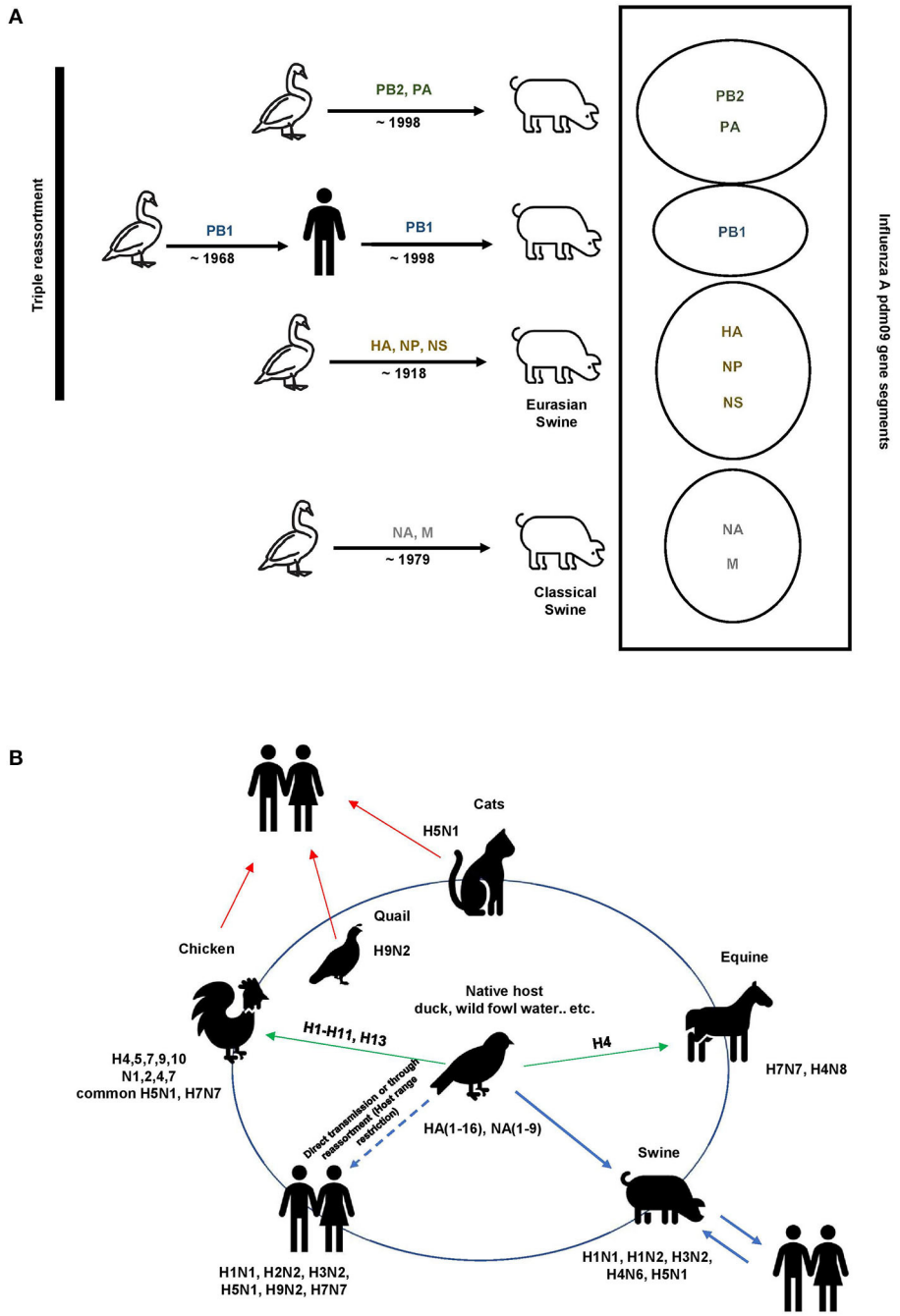


FIGURE 2 | Influenza A evolution. **(A)** Triple reassortment influenza A viruses of the H1N1 subtype containing avian, swine, and human gene segments. The colored solid genes represent the gene segments as follows: yellow, classical swine A (H1N1) virus; green, North American avian virus; blue, human A (H3N2) virus; gray, Eurasian avian-like swine A(H1N1). **(B)** Reservoirs and interspecies transmission events of the pathogenic influenza A viruses. Wild birds, domestic birds, pigs, horses, and humans maintain their influenza A viruses. Spillover events occasionally occur, most frequently from wild birds (arrows in green).

that have previously been confronted, potentially causing another epidemic/pandemic (38).

SARS-CoV

In the early stages of the SARS outbreak, most of the new patient cases had animal exposure before developing the disease. Wide-ranging investigations revealed that SARS-CoV strains were transmitted to palm civets from other animals (39–41). Later, two studies reported the discovery of coronaviruses related to human SARS-CoV, which were named SARS-like coronaviruses or SARSr-CoVs, in horseshoe bats (genus *Rhinolophus*) (42, 43). Another study revealed that the viral

strains of the SARS-like coronaviruses contain all of the genetic elements that are needed to form SARS-CoV. In particular, the bat strain WIV16, the closest relative to SARS-CoV, likely occurred through recombination of two other prevalent bat SARSr-CoV strains. These results suggest that bats may be the natural reservoirs for the virus and that palm civets are only intermediate hosts (**Supplementary Figure 1**) (44, 45).

Thus, the hypothesis formed was that the direct ancestor of SARS-CoV was produced by recombination within bats and then transmitted to palm civets or other mammals via fecal–oral transmission. When virus-infected civets were transported to Guangdong market, the virus spread among the civets in the market and underwent further mutations before transmission to humans (46).

MERS-CoV

Unlike the SARS cases, most of the MERS cases had previous contact with dromedary camels. The MERS-CoV strains isolated from camels were almost identical to those isolated from humans (47, 48), and the MERS-CoV isolates were found to be highly prevalent in camels from the Middle East, Africa, and Asia (49, 50). Genomic sequence analysis indicated that the *Tylonycteris* bat coronaviruses HKU4 and HKU5 are phylogenetically related to MERS-CoV (they are all representatives of betacoronavirus lineage C) (51). Generally, all of the related MERS-CoVs isolated from bats support the hypothesis that MERS-CoV originated from bats (**Supplementary Figure 1**) (46).

SARS-CoV-2

Before the epidemic outbreak of COVID-19 in late January 2020, several patients had been exposed to different animals (from wild animals to poultry) at the Huanan seafood wholesale market. When the CDC declared the situation to be an epidemic, several studies identified potential reservoirs, but at present, the origin and evolution of SARS-CoV-2 remain debatable. The earliest genomic sequence analysis of SARS-CoV-2 indicated that it is a member of the genus *Betacoronavirus* and falls within the subgenus *Sarbecovirus*, which also includes SARS-CoV (9, 35, 52–54). As mentioned above, preliminary comparisons revealed that SARS-CoV-2 has an almost 79% similarity with SARS-CoV at the nucleotide sequence level and a 96% similarity with horseshoe bat RaTG13 (55–57). Correspondingly, a comparative study between the RmYN02 virus from *Rhinolophus* bats in Yunan Province, China, and SARS-CoV-2 indicated that RmYN02 was the closest relative to the long replicase gene of SARS-CoV-2 (∼97% nucleotide sequence similarity) (35, 36).

Even though bats are likely to be the reservoir host for this virus, their general biological differences from humans make it feasible that other mammalian species acted as intermediate hosts, in which SARS-CoV-2 obtained some or all of the mutations needed for effective human transmission. One of the suspected intermediate hosts, the Malayan pangolin, harbors coronaviruses showing high similarity to SARS-CoV-2 in the receptor-binding domain, which contains mutations believed to promote binding to the angiotensin-converting enzyme 2 (ACE2) receptor and demonstrates a 97% amino acid sequence similarity. By contrast, the genomic similarity was more divergent from SARS-CoV-2 (∼91%) at the whole genome level (**Supplementary Figure 1**) (58, 59).

Coronaviruses have lower mutation rates than other RNA viruses, especially influenza A viruses, and high rates of viral replication within hosts because of the 3′-to-5′ exoribonuclease activity associated with the nonstructural protein nsp.14 (36, 60). This protein has an RNA proofreading function and is responsible for coronaviruses' resistance to RNA mutagens (60, 61).

RECEPTOR BINDING OF VIRUSES

The high unpredictability among influenza A viral strains and their HAs relates to the significant discrepancy among host cells in showing different vulnerabilities to viral infection. HA plays a role in mediating the binding of influenza A viruses to sialic acid host cell receptors (62). The receptor-binding site lies at the top of the R domain of HA and contains exceptionally variable antigenic binding loops (63). Once the virus is bound to the host receptor, endocytosis of the virus element occurs. Additionally, a pH-dependent membrane fusion process is significant in controlling the viral genome's release into the host cell. Influenza A viral strains and their HAs are very variable, which contributes to the significantly different vulnerabilities of host cells to viral infection (64).

Influenza A viruses have demonstrated dominant genomic mutations, such as those within the HA 220 loop (Q223) and the D222G and D222N mutations, in which aspartic acid (D) is replaced by glycine (G) or asparagine (N), respectively. The D222G mutation is responsible for a change in receptor-binding affinity that enables the virus to bind to α-2,6 and α-2,3 sialic acid receptors on the epithelial cells of the upper respiratory tract and ciliated epithelial cells in the lower respiratory tract, respectively (65, 66).

Although HA plays a crucial role in receptor binding and concurrent mutation capabilities, NA also has a key role in removing sialic acids from cellular receptors and from the new HA and NA on budding virions, which are sialylated as part of the glycosylation processes within the host cell (67). A balance between HA and NA is essential for viral fitness. Any mutations in HA or environmental changes, such as low pH conditions, can affect NA's activity against sialoglycans (68, 69).

The SARS-CoV trimeric spike protein facilitates coronavirus entry into host cells by binding to the host receptor and subsequently fusing the viral and host membranes. The spike protein consists of three segments, one of which is the ectodomain (70). The ectodomain is composed of two subunits: S1 and S2. The S1 subunit contains two individual domains, an N-terminal domain (NTD) and a C-domain, and each NTD or C-domain (sometimes both) binds to the host receptor to function as the receptor-binding domain (RBD). ACE2 is the host cell receptor of SARS-CoV and the primary target of deactivating antibodies. Several studies have shown that the binding affinity between the RBD of each SARS-CoV strain and ACE2 positively correlates with the contagion of different SARS-CoV strains in host cells (**Supplementary Figure 2**) (71, 72).

The MERS-CoV spike protein subunit S1 C-domain has also been identified as the RBD (73). However, unlike SARS-CoV, MERS-CoV uses a dipeptidyl peptidase 4 (DPP4) β-propeller as its receptor. Likewise, the RBD of MERS-CoV contains an accessory subdomain that functions as the receptor-binding motif (RBM). Although the RBD core structures are remarkably analogous between MERS-CoV and SARS-CoV, their RBMs are distinct and may result in the recognition of different receptors (**Supplementary Figure 2**) (73).

Since the outbreak of SARS-CoV-2, several studies have analyzed its genome and compared it with other coronaviruses, such as MERS-CoV and SARS-CoV (74, 75). The results of these studies have shown that SARS-CoV-2 has a similar RBD structure to that of SARS-CoV, despite amino acid variations at some key residues (9). Genomic comparison of SARS-CoV-2 with SARS-CoV and bat SARS-like coronaviruses revealed that the S1 subunits of the spike proteins have a sequence identity of ∼75%, and recent experimental studies confirmed that ACE2 is the human receptor of SARS-CoV-2 (34). Therefore, it is essential to characterize the human receptor-binding capacity of SARS-CoV-2 to evaluate its human–human transmissibility. A recent study used the protein–protein docking method to measure the interaction between the SARS-CoV-2 spike RBD and ACE2; it was revealed that the SARS-CoV-2 human receptor-binding affinity was 73% of that of SARS-CoV, which suggests that SARS-CoV-2 binds to ACE2 with intermediate affinity (76) (**Supplementary Figure 2**).

HOST FACTORS, DISEASE SEVERITY, AND PATHOGENESIS

Influenza, SARS, and MERS have caused major global health threats, and now the COVID-19 pandemic is rapidly spreading worldwide and is having a widespread and profound impact. Both viral and host factors determine the severity and clinical outcomes of the diseases caused by these viruses. Host factors include host immunity, age, sex, morbidities, and genetic variations.

Influenza infections can cause high morbidity and mortality rates in the elderly (65 or older) and young populations with comorbidities (**Figure 1C**). Pathogenesis following influenza A infection occurs in two stages. The first stage is defined by the peak viral titer, along with the peak amount of inflammation associated with the infection, and lasts ∼1 to 3 days. In the second stage, the infection progresses in some patients, and in severe cases, it may be associated with acute respiratory distress syndrome and sometimes death (77). Once a patient is infected with an influenza A virus, the humoral immune response will release neutralizing antibodies to target the influenza HA protein by blocking the binding of HA to sialic acids, thereby preventing viral fusion, inhibiting the release of offspring virions, and delaying proteolytic cleavage of HA by host receptors (78).

Once a patient is infected with SARS-CoV, MERS-CoV, or SARS-CoV-2, the host innate immune system will identify the virus by using pattern recognition receptors, such as a toll-like receptor, NOD-like receptor, or RIG-I-like receptor, to recognize pathogen-associated molecular patterns. The adaptive immune response also plays a significant antiviral role by stabilizing the host defense mechanism against pathogens and minimizing the risk of developing an autoimmune reflex response or inflammation (9, 79). In general, human coronaviruses can be classified into two types: lowly pathogenic and highly pathogenic. Viruses with low pathogenicity, including HCoV-229E, HCoV-OC43, HCoV-NL63, and HCoV-HKU, can cause mild upper respiratory tract infections. In contrast, highly pathogenic viruses, including SARS-CoV, MERS-CoV, and SARS-CoV-2, can cause lower respiratory tract infections, severe pneumonia, and sometimes fatal acute lung injury or acute respiratory distress syndrome, especially in older individuals (≥65 years old) (**Figure 1C**) (80).

In addition to the lungs, coronavirus infection may damage other organs or tissues, including the gastrointestinal tract (81), spleen, lymph nodes, brain, skeletal muscles, thyroid, and heart (82, 83). The destruction of lung cells prompts a local immune response, engaging macrophages and monocytes that respond to the infection, release cytokines, and enhance adaptive T and B cell immune responses. In some cases, a dysfunctional immune response occurs, which can cause severe lung and systemic pathology. The invading coronavirus may incite host immune responses, and an excessive immune response may cause immunopathological damage (known as a cytokine storm) in patients with coronavirus infections (9, 84). Cytokine storms may enhance the infiltration of non-neutralizing antiviral proteins that facilitate viral entry into host cells, leading to increased viral infectivity (82, 85). Therefore, cytokine storms play a key role in the pathogenesis and clinical outcomes of patients with coronavirus infection.

TRANSMISSIBILITY AND VIRULENCE

The initiation of a pandemic requires the rise of a virus in a human population in which there is little or no pre-existing immunity, and the virus must be able to persist through human-to-human transmission (86, 87). The ability of influenza A viruses to adapt to various hosts and undergo reassortment events ensures the constant generation of new strains. These strains have variable degrees of pathogenicity, pandemic transmissibility, and reproduction numbers (R_0) (**Table 1**) (88). However, only three subtypes of influenza A (H1–H3) have acquired the properties to cause pandemics in the last two centuries. Thus, an understanding of the capability of a virus to attain a contagious phenotype is a critical factor in evaluating the pandemic potential of novel subtypes (89, 90). The use of animal models has facilitated detailed studies of influenza A virus transmission by the contact and respiratory droplet routes. The presence of a single sick individual in a small space, such as an airplane or room, has been shown to be adequate for an outbreak among healthy individuals (**Supplementary Figure 3**) (91). Although infection and case fatality rates vary from one pandemic to another, the rates of influenza A virus infections in the pandemics were high, especially among people with little to no pre-existing immunity. When pandemic viruses become

established in humans, their effective seasonal spread among healthy individuals eventually provides an enduring and even more significant public health issue in terms of hospitalizations and, in some cases, fatalities. Particle size (92), the distance of spread (92), disposition (92, 93), temperature (94), and relative humidity (95) are all considered to be factors that influence the rate of transmissibility of influenza A viruses. In addition, sialic acid receptors (α-2,3 and α-2,6) can affect the general species-specific cellular tropism of influenza A viruses (63).

Contaminated surfaces also play an essential role in transmission. A respiratory pathogen can survive on surfaces, be transferred to hands or other equipment, and initiate infection through contact with the eyes, nose, or mouth (**Supplementary Figure 3**) (96). Influenza A has been shown to survive for 24–48 h on stainless steel and plastic surfaces. Inversely, the strains survived for <8–12 h on cloth, paper, and tissues. Quantifiable amounts of influenza A viruses were observed to be transmitted from stainless steel surfaces to hands after 24 h and from tissues to hands for up to 15 min. Viruses also survive on hands for up to 5 min after transfer from environmental surfaces. These results indicate a high transmission rate for influenza A viruses (97).

SARS-CoV, MERS-CoV, and SARS-CoV-2 can survive on surfaces for extended periods, sometimes up to months. Like the influenza A viruses, the factors affecting the survival of these viruses on surfaces include the strain variation, titer, surface type, mode of deposition, temperature, humidity, and method used to determine the viability of the virus (98, 99). Several studies have indicated that SARS-CoV, MERS-CoV, and SARS-CoV-2 can survive on dry surfaces for a sufficient duration to accelerate onward transmission. Viable MERS-CoV was detected on steel and plastic surfaces after 48 h at 20°C with 40% relative humidity, with a decreased viability of about 8 h at 30°C with 80% relative humidity and of about 24 h at 30°C with 30% relative humidity. The estimated half-life of MERS-CoV ranges from ∼0.5 to 1 h (98). On the other hand, another study conducted on the viability of SARS-CoVs detected on plastic surfaces and on polystyrene Petri dishes revealed that the virus survived for more than 5 days and more than 20 days, respectively, at room temperature. The viral viability was constant at lower temperatures (28°C) and lower humidity (80–89%) (100), whereas survival times ranged from 5 min to 2 days on paper, disposable gowns, and cotton gowns (99).

Since the SARS-CoV-2 outbreak began, several researchers have attempted to analyze the survival time of this virus on different surfaces. One study published in the middle of March 2020 analyzed the aerosol and surface stabilities of SARS-CoV-2 and SARS-CoV. The study utilized five different environments (aerosols, plastic, stainless steel, copper, and cardboard). The results showed that the half-lives of SARS-CoV-2 and SARS-CoV were similar in aerosols and on copper. However, on cardboard surfaces, the half-life of SARS-CoV-2 was longer than that of SARS-CoV, and the highest levels of viability for both viruses were observed on stainless steel and plastic (∼5.6 h on stainless steel and 6.8 h on plastic). The researchers concluded that the differences in the epidemiological characteristics of these viruses could result from other factors and that aerosol and fomite transmission of SARS-CoV-2 is probable because the virus can remain viable and infectious in aerosols and on surfaces for hours and hours to days, respectively (101).

The effective management and control of such infections are increasingly performed with extensive contributions from mathematical modeling, which not only provides information on the nature of the infection itself but also makes predictions about the likely outcome of alternative courses of action (102). One useful mathematical model is the reproductive number R_0, which is defined as the average number of secondary cases generated per typical infectious case (103). A value of $R_0 > 1$ indicates that the infection may persist or grow in the population, whereas a value of $R_0 < 1$ indicates that this infection will decrease in the population, although exceptions occur (103). The majority of seasonal influenza R_0 values have been calculated for different populations and different continents, such as Europe and North America, with a median point estimate of $R_0 = 1.27$ (IQR: 1.19–1.37) (104). The initial estimations of the reproduction numbers of SARS-CoV and MERS-CoV were calculated for China and the Middle East with R_0 median = 0.58 (IQR: 0.24–1.18) (105) and R_0 mean = 0.69 (95% CI: 0.50–0.92) (106), respectively. However, among the four viruses, SARS-CoV-2 has been calculated to be the most contagious, such as the R_0 value associated with the Italian outbreak with a median point estimate of $R_0 = 3.1$ (coefficient of determination, $r^2 = 0.99$) (107).

PREVENTION, CONTROL, AND TREATMENT OF VIRUS INFECTION

Strategies for preventing and controlling pandemic/epidemic viruses can be improved by being well-prepared. Preparedness strategies, which primarily include the quarantine of infected persons, self-protection (wearing facemasks, using disinfectants, washing hands, and disinfecting surfaces with bleach or alcohols), and social distancing are all considered to be important for a comprehensive plan that can be tested and promoted by conducting exercises to engage the whole of society.

An influenza pandemic can be catastrophic, and in a typical year of seasonal outbreaks, influenza A viruses cause as many as 5 million cases of severe illness in humans and over 500,000 deaths. After the first confirmed cases of H1N1 influenza appeared in Mexico in February 2009, cases began to spread to the United States, and by the end of April 2009, cases had been reported in several United States cities and other countries on various continents, such as Canada, the United Kingdom, and New Zealand (108). During the last pandemic, the first activation of the International Health Regulations (IHR) provisions was prompted. The discussions that led to the IHR implementation were based on the SARS outbreak experience in 2003. These regulations describe the responsibilities of individual countries and the leadership role of the WHO in declaring and managing a public health emergency of international concern, establishing systematic approaches to surveillance, promoting technical cooperation, and sharing logistic support

TABLE 2 | List of antiviral drugs and vaccine approaches for SARS-CoV-2, SARS-CoV, MERS-CoV, and influenza viruses.

Virus	Drug or vaccine	Drug mechanism of action/comments
SARS-CoV-2	Bevacizumab, Chloroquine phosphate, Methylprednisolone, Fingolimod, Favipiravir, Lopinavir and ritonavir, Remdesivir, mRNA-1273*, ChAdOx1 nCoV-19*	• **Bevacizumab:** inhibiting vascular endothelial growth factor (VEGF), which is higher in COVID-19 patients than in healthy controls; VEGF is the most potent vascular permeability inducer that induces hypoxia and severe inflammation • **Chloroquine:** increasing endosomal pH, which is required for virus fusion; interfering with the glycosylation of cellular receptors of SARS-CoV; suppressing the production or release of tumor necrosis factor α and interleukin 6 • **Glucocorticoids:** suppressing "cytokine storms" • **Fingolimod:** preventing acute respiratory distress syndrome development • **Favipiravir:** based on the results of two trials conducted in Wuhan and Shenzhen, China recommended this drug as a treatment approach for COVID-19 • **Lopinavir/ritonavir:** reducing viral replications in patients infected with SARS and MERS; ritonavir reduces the first pass metabolism of lopinavir to increase its bioavailability • **Remdesivir:** antiviral drug against a wide array of RNA viruses that works by combining with the nascent viral RNA chains to result in premature termination, reducing virus infections (82, 117–122)
SARS-CoV	Ribavirin, Methylprednisolone, Interferons, Lopinavir and ribavirin, Pentaglobin*	• **Ribavirin:** preventing replication of RNA and DNA viruses • **Methylprednisolone:** using interferons plus corticosteroids to reduce disease-associated impaired oxygen saturation, radiographic lung abnormalities, and creatine kinase levels (controversial arguments about using corticosteroids in SARS) • **Interferons:** reducing viral replication • **Lopinavir and ribavirin:** blocking the final step of virion assembly; reducing the peak viral load and the associated immunopathological damage (123)
MERS-CoV	Ribavirin and interferon-α2a, Lopinavir/ritonavir, Convalescent plasma*	• **Ribavirin:** combining interferon-α2b to reduce MERS-CoV replication • **Lopinavir/ritonavir:** improving the outcomes of MERS-CoV infection; improving pulmonary function but not reducing virus replication or severe lung pathology (124)
Influenza A virus (drugs recommended by CDC to treat flu in the 2019–2020 season)	Oseltamivir phosphate, Zanamivir, Peramivir, Baloxavir marboxil, flu vaccines (such as flu shots, nasal spray flu vaccine, quadrivalent influenza)	• **Oseltamivir:** blocking neuraminidases on the surfaces of influenza viruses; interfering with host cell release of complete viral particles • **Zanamivir:** inhibiting influenza A and B virus neuraminidases; preventing the release of progeny viruses from host cell surfaces; inhibiting viral replication • **Peramivir:** inhibiting influenza virus neuraminidases • **Baloxavir marboxil:** inhibiting polymerase acidic endonuclease, an enzyme essential for viral replication; being a prodrug converted by the hydrolysis of baloxavir • **Flu vaccines:** including flu shots, nasal spray flu vaccine (FluMist Quadrivalent), quadrivalent influenza vaccine, flu vaccination by jet injector, Fluzone high-dose seasonal influenza vaccine, flu vaccine with adjuvant (FLUAD), cell-based flu vaccines (Flucelvax Quadrivalent), recombinant influenza vaccine, and intradermal influenza vaccination (125, 126).

**Indicates that the drug is under investigation; otherwise, it has been approved by the FDA.*

(108). However, because of the significant diversity of influenza viruses in animal hosts, extensive experimental testing and the development of pandemic preparedness measures against all viruses is unachievable (109).

In this regard, the WHO periodically updates the influenza risk management and preparedness plan, and the latest guidance document, *Pandemic Influenza Risk Management* (PIRM), was released in May 2017 (110). This updated document supports national and global pandemic preparedness and risk management and utilizes lessons learned at the country, regional, and global levels (110). Furthermore, several WHO preparedness documents have been released since PIRM, such as *Essential steps for developing or updating a national pandemic influenza preparedness plan* (released in March 2018) and *A practical guide for developing and conducting simulation exercises to test and validate pandemic influenza preparedness plans* (published in September 2018) (111).

During the SARS epidemic, more than 8,000 people were infected, and 774 deaths occurred between November 2002 and December 2003. SARS is highly contagious and is transmitted primarily by respiratory droplets; the highest transmission rates of SARS occurred in healthcare facilities (112). At the end of the SARS outbreak, the cases of over 1,700 healthcare workers who had been affected were reported to the WHO, from China (19% of total cases), Canada (43%), France (29%), and Hong Kong (22%). During this epidemic, insufficient or inappropriate infection control measures, such as inconsistent use of personal protective equipment, reuse of N95 masks, and lack of adequate infection control, were related to the high risk of infection among healthcare workers (113). Thus, in 2004, after the epidemic was contained, the WHO released a framework that was prepared according to the six phases of an epidemic, moving from preparedness, planning, and routine surveillance for cases, through to the prevention of the consequent international spread, to the disruption of global transmission (114).

Since 2012, 27 countries have reported cases of MERS; Saudi Arabia has reported ∼80% of human cases, and more than 50% of the cases in healthcare workers were nurses (115). The WHO, in collaboration with the Food and Agriculture Organization of the United Nations (FAO), the World Organization for Animal Health (OIE), and national governments, have been working with healthcare workers and scientists in affected countries to gather and share scientific evidence based on the previous coronavirus epidemic. This information gathering process has been beneficial for better understanding of the virus and the disease it causes

and for the regulation of outbreak response priorities, treatment approaches, and clinical management tactics (113).

Although accumulated knowledge and risk preparedness from the influenza pandemics and SARS/MERS epidemics allowed researchers to examine the effectiveness of strategic plans in dealing with the ongoing pandemic of COVID-19, several challenges have been raised in preventing the spread of COVID-19, such as the lack of medical supplies and laboratory facilities for the assessment of the disease and the presentation of a high number of asymptomatic cases. In response to the announcement of the emergency, governments were bound by the IHR to disclose vital information regarding the identification and detection of COVID-19, regardless of the causative agent. Within the context of the Global Humanitarian Response Plan, a Health Cluster platform has been created to assess the response to the COVID-19 pandemic worldwide. This framework has adopted the following strategies: contain the spread of the COVID-19 pandemic and decrease morbidity and mortality; decrease the deterioration of human assets and rights, social cohesion, and livelihoods; and protect, assist, and advocate for refugees, internally displaced people, migrants, and host communities who are particularly vulnerable to the pandemic (source: WHO). The primary goal of the Health Cluster is to coordinate and support partners to fulfill essential health services to achieve the framework strategies. This goal is achieved by different roles and tasks, such as by raising awareness, alertness, and response planning at the country level and by conducting training and simulation exercises. The WHO Health Cluster framework is a gateway to useful resources to support COVID-19 preparedness and response (116).

Generally, each pandemic/epidemic has presented a public health emergency of uncertain scope and effect; thus, essential elements of current approaches to pandemic preparedness and extenuation, such as the development of vaccines and stockpiling of antiviral drugs, necessitate detailed virological and immunological data on viruses with apparent pandemic potential. However, the development of vaccines against new strains is challenging. Therefore, physicians and health workers have found themselves facing the massive challenge of preventing infections or stabilizing patients' conditions. Thus, several promising attempts have been made to utilize different antiviral treatments that have already been approved by the U.S. Food and Drug Administration (FDA) for the treatment of viral pneumonia infections. A list of antiviral drugs and vaccine approaches for influenza viruses, SARS-CoV, MERS-CoV, and SARS-CoV-2 that have been used in clinics or are undergoing clinical trials are summarized in **Table 2**.

DISCUSSION AND CONCLUSION

Although the mode of transmission for SARS-CoV-2 is still somewhat unclear, all four viruses are thought to be transmitted by the same mechanism. Infection via respiratory droplets or secretions of infected individuals is the primary mode of transmission between humans. The spread of infection is occurring more rapidly for the current outbreak than in the SARS and MERS epidemics, although rates of human-to-human transmission were generally lower for MERS.

The CFRs across the four viruses range from 0.1 to 35% (**Table 1**), with the highest rate for MERS cases and the lowest for seasonal influenza; however, it is essential to note that the CFR for COVID-19 should be interpreted carefully because the outbreak is still ongoing.

With the exception of the influenza A viruses, the other viruses (SARS-CoV, MERS-CoV, and SARS-CoV-2) are similar in zoonotic transmission. The MERS-CoV reservoir hosts are dromedary camels, and the SARS-CoV reservoir hosts are likely bats. It is still unclear whether SARS-CoV-2 was zoonotically transmitted from an infected palm civet, snake, or other animal at the Chinese seafood market.

Regarding the origin of the virus, SARS-CoV and SARS-CoV-2 originate from China and share a high degree of similarity, including exposure to wild animals, whereas MERS-CoV and SARS-CoV-2 have shared similarities in that cases can remain asymptomatic while still spreading the disease. Furthermore, influenza A viruses and SARS-CoV-2 also have a similar characteristic when it comes to transmissibility (127).

In the setting of extensive SARS-CoV-2 transmissions, the possibility of SARS-CoV-2 should be considered in all persons with a fever or lower respiratory infection, because it is challenging to straightforwardly distinguish between seasonal influenza and COVID-19, even if an epidemiologic link cannot be readily established. Furthermore, the timely reporting of cases, updates on clinical status and disposition of patients, the real-time analysis of data, and the appropriate dissemination of information are essential for outbreak-managing decisions.

AUTHOR CONTRIBUTIONS

ZA: conceptualization, methodology, investigation, writing—original draft, and visualization. ML: visualization. XW: conceptualization, methodology, project administration, funding acquisition, writing—review and editing, and supervision. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the China Pharmaceutical University (grant number 3150120001 to XW).

ACKNOWLEDGMENTS

We thank China Pharmaceutical University for its support and funding.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.552909/full#supplementary-material

Supplementary Figure 1 | The origins and intermediate hosts of SARS-CoV-2, SARS-CoV, and MERS-CoV.

Supplementary Figure 2 | Virus-host interaction. Th1, T helper 1; Th17, T helper 17; ACE2, angiotensin-converting enzyme 2; INF-1, interferon 1; INFγ, interferon

gamma; DPP4, dipeptidyl peptidase-4; HA, hemagglutinin; NA, neuraminidase; M2e, Matrix 2 protein; MHC-1, major histocompatibility complex class 1.

Supplementary Figure 3 | Potential transmission routes of respiratory infection between infected and susceptible individuals (128). Respiratory infections with a droplet nuclei size $\leq 5\,\mu m$ can travel to a distance ≥ 1 m. In contrast, respiratory infections with a droplet nuclei size $\geq 5\,\mu m$ cannot travel to a distance ≥ 1 m. Large droplets may fall on different surfaces and infect healthy individuals through direct or indirect contact.

REFERENCES

1. Jordan D. *The Deadliest Flu: The Complete Story of the Discovery and Reconstruction of the 1918 Pandemic Virus.* Centers for Disease Control and Prevention, National Center for Immunization and Respiratory Diseases (NCIRD), December 17 (2019). Available online at: https://www.cdc.gov/flu/pandemic-resources/1918-pandemic-h1n1.html (accessed March 19, 2020).
2. Glezen WP. Emerging infections: pandemic influenza. *Epidemiol Rev.* (1996) 18:64–76. doi: 10.1093/oxfordjournals.epirev.a017917
3. Viboud C, Grais RF, Lafont BA, Miller MA, Simonsen L. Multinational impact of the 1968 Hong Kong influenza pandemic: evidence for a smoldering pandemic. *J Infect Dis.* (2005) 192:233–48. doi: 10.1086/431150
4. Garten RJ, Davis CT, Russell CA, Shu B, Lindstrom S, Balish A, et al. Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. *Science.* (2009) 325:197–201. doi: 10.1126/science.1176225
5. Shieh WJ, Blau DM, Denison AM, Deleon-Carnes M, Adem P, Bhatnagar J, et al. 2009 pandemic influenza A (H1N1): pathology and pathogenesis of 100 fatal cases in the United States. *Am J Pathol.* (2010) 177:166–75. doi: 10.2353/ajpath.2010.100115
6. Peiris JSM, Yuen KY, Osterhaus ADME, Stöhr K. The severe acute respiratory syndrome. *N Engl J Med.* (2003) 349:2431–41. doi: 10.1056/NEJMra032498
7. Zumla A, Hui DS, Perlman S. Middle East respiratory syndrome. *Lancet.* (2015) 386:995–1007. doi: 10.1016/S0140-6736(15)60454-8
8. The Lancet. Emerging understandings of 2019-nCoV. *Lancet.* (2020) 395:311. doi: 10.1016/S0140-6736(20)30186-0
9. Lu R, Zhao X, Li J, Niu P, Yang B, Wu H, et al. Genomic characterisation and epidemiology of 2019 novel coronavirus: implications for virus origins and receptor binding. *Lancet.* (2020) 395:565–74. doi: 10.1016/S0140-6736(20)30251-8
10. Xiao K, Zhai J, Feng Y, Zhou N, Zhang X, Zou JJ, et al. Isolation of SARS-CoV-2-related coronavirus from Malayan pangolins. *Nature.* (2020) 583:286–9. doi: 10.1038/s41586-020-2313-x
11. Wang H, Yang P, Liu K, Guo F, Zhang Y, Zhang G, et al. SARS coronavirus entry into host cells through a novel clathrin- and caveolae-independent endocytic pathway. *Cell Res.* (2008) 18:290–301. doi: 10.1038/cr.2008.15
12. Millet JK, Whittaker GR. Host cell entry of Middle East respiratory syndrome coronavirus after two-step, furin-mediated activation of the spike protein. *Proc Natl Acad Sci USA.* (2014) 111:15214–9. doi: 10.1073/pnas.1407087111
13. Lakadamyali M, Rust MJ, Zhuang X. Endocytosis of influenza viruses. *Microbes Infect.* (2004) 6:929–36. doi: 10.1016/j.micinf.2004.05.002
14. Xu Z, Shi L, Wang Y, Zhang J, Huang L, Zhang C, et al. Pathological findings of COVID-19 associated with acute respiratory distress syndrome. *Lancet Respir Med.* (2020) 8:420–2. doi: 10.1016/S2213-2600(20)30076-X
15. Yang M, Hon KL, Li K, Fok TF, Li CK. The effect of SARS coronavirus on blood system: its clinical findings and the pathophysiologic hypothesis. *Zhongguo shi Yan Xue Ye Xue Za Zhi.* (2003) 11:217–21.
16. Park GE, Kang CI, Ko JH, Cho SY, Ha YE, Kim YJ, et al. Differential cell count and CRP level in blood as predictors for middle east respiratory syndrome coronavirus infection in acute febrile patients during nosocomial outbreak. *J Korean Med Sci.* (2017) 32:151–4. doi: 10.3346/jkms.2017.32.1.151
17. Perera RA, Wang P, Gomaa MR, El-Shesheny R, Kandeil A, Bagato O, et al. Seroepidemiology for MERS coronavirus using microneutralisation and pseudoparticle virus neutralisation assays reveal a high prevalence of antibody in dromedary camels in Egypt, June 2013. *Euro Surveill.* (2013) 18:20574. doi: 10.2807/1560-7917.ES2013.18.36.20574
18. Lee N, Le Sage V, Nanni AV, Snyder DJ, Cooper VS, Lakdawala SS. Genome-wide analysis of influenza viral RNA and nucleoprotein association. *Nucleic Acids Res.* (2017) 45:8968–77. doi: 10.1093/nar/gkx584
19. *Influenza A Model Receives A Face-Lift*. (2020). Available online at: https://www.contagionlive.com/news/influenza-a-model-receives-a-face-lift
20. Vincent A, Awada L, Brown I, Chen H, Claes F, Dauphin G, et al. Review of influenza A virus in swine worldwide: a call for increased surveillance and research. *Zoonoses Public Health.* (2014) 61:4–17. doi: 10.1111/zph.12049
21. Tian J, Zhang C, Qi W, Xu C, Huang L, Li H, et al. Genome sequence of a novel reassortant H3N2 avian influenza virus in southern China. *J Virol.* (2012) 86:9553–4. doi: 10.1128/JVI.01523-12
22. Bouvier NM, Palese P. The biology of influenza viruses. *Vaccine.* (2008) 26(Suppl. 4):D49–53. doi: 10.1016/j.vaccine.2008.07.039
23. Ahn I, Jeong BJ, Bae SE, Jung J, Son HS. Genomic analysis of influenza A viruses, including avian flu (H5N1) strains. *Eur J Epidemiol.* (2006) 21:511–9. doi: 10.1007/s10654-006-9031-z
24. Yoon SW, Webby RJ, Webster RG. Evolution and ecology of influenza A viruses. *Curr Top Microbiol Immunol.* (2014) 385:359–75. doi: 10.1007/82_2014_396
25. Reperant LA, Kuiken T, Osterhaus ADME. Influenza viruses. *Hum Vaccines Immunother.* (2012) 8:7–16. doi: 10.4161/hv.8.1.18672
26. Anderson TK, Macken CA, Lewis NS, Scheuermann RH, Van Reeth K, Brown IH, et al. A phylogeny-based global nomenclature system and automated annotation tool for H1 hemagglutinin genes from swine influenza A viruses. *mSphere.* (2016) 1:e00275-16. doi: 10.1128/mSphere.00275-16
27. Guarnaccia T, Carolan LA, Maurer-Stroh S, Lee RT, Job E, Reading PC, et al. Antigenic drift of the pandemic 2009 A(H1N1) influenza virus in A ferret model. *PLoS Pathog.* (2013) 9:e1003354. doi: 10.1371/journal.ppat.1003354
28. Tewawong N, Prachayangprecha S, Vichiwattana P, Korkong S, Klinfueng S, Vongpunsawad S, et al. Assessing antigenic drift of seasonal influenza A(H3N2) and A(H1N1)pdm09 viruses. *PloS ONE.* (2015) 10:e0139958. doi: 10.1371/journal.pone.0139958
29. Pellett PE, Mitra S, Holland TC. Chapter 2 - basics of virology. In: Tselis AC, Booss J, editors. *Handbook of Clinical Neurology*. 123: Michigan City, IN: Elsevier (2014). p. 45–66.
30. Torres J, Maheswari U, Parthasarathy K, Ng L, Liu DX, Gong X. Conductance and amantadine binding of a pore formed by a lysine-flanked transmembrane domain of SARS coronavirus envelope protein. *Prot Sci.* (2007) 16:2065–71. doi: 10.1110/ps.062730007
31. Tan Y-J, Lim SG, Hong W. Understanding the accessory viral proteins unique to the severe acute respiratory syndrome (SARS) coronavirus. *Antiviral Res.* (2006) 72:78–88. doi: 10.1016/j.antiviral.2006.05.010
32. Chung YS, Kim JM, Man Kim H, Park KR, Lee A, Lee NJ, et al. Genetic characterization of middle east respiratory syndrome coronavirus, South Korea, (2018) *Emerg Infect Dis.* (2019) 25:958–62. doi: 10.3201/eid2505.181534
33. Grifoni A, Weiskopf D, Ramirez SI, Mateus J, Dan JM, Moderbacher CR, et al. Targets of T cell responses to SARS-CoV-2 coronavirus in humans with COVID-19disease and unexposed individuals. *Cell.* (2020) 181:1489–501.e15. doi: 10.1016/j.cell.2020.05.015
34. Walls AC, Park YJ, Tortorici MA, Wall A, McGuire AT, Veesler D. Structure, function, and antigenicity of the SARS-CoV-2 spike glycoprotein. *Cell.* (2020) 181:281–92.e6. doi: 10.1016/j.cell.2020.02.058
35. Chen J, Liu D, Liu L, Liu P, Xu Q, Xia L, et al. A pilot study of hydroxychloroquine in treatment of patients with common coronavirus disease-19 (COVID-19). *J Zhejiang Univ Med Sci.* (2020) 49:215–19. doi: 10.3785/j.issn.1008-9292.2020.03.03
36. Andersen KG, Rambaut A, Lipkin WI, Holmes EC, Garry RF. The proximal origin of SARS-CoV-2. *Nat Med.* (2020) 26:450–2. doi: 10.1038/s41591-020-0820-9
37. Brown IH. History and epidemiology of Swine influenza in Europe. *Curr Top Microbiol Immunol.* (2013) 370:133–46. doi: 10.1007/82_2011_194
38. Castelán-Vega JA, Magaña-Hernández A, Jiménez-Alberto A, Ribas-Aparicio RM. The hemagglutinin of the influenza A(H1N1)pdm09 is mutating towards stability. *Adv Appl Bioinform Chem.* (2014) 7:37–44. doi: 10.2147/AABC.S68934

39. Guan Y, Zheng BJ, He YQ, Liu XL, Zhuang ZX, Cheung CL, et al. Isolation and characterization of viruses related to the SARS coronavirus from animals in southern China. *Science.* (2003) 302:276–8. doi: 10.1126/science.1087139
40. Tu C, Crameri G, Kong X, Chen J, Sun Y, Yu M, et al. Antibodies to SARS coronavirus in civets. *Emerg Infect Dis.* (2004) 10:2244–8. doi: 10.3201/eid1012.040520
41. Wang M, Xu HF, Zhang ZB, Zou XZ, Gao Y, Liu XN, et al. [Analysis on the risk factors of severe acute respiratory syndromes coronavirus infection in workers from animal markets]. *Zhonghua Liu Xing Bing Xue Za Zhi.* (2004) 25:503–5.
42. Lau SK, Woo PC, Li KS, Huang Y, Tsoi HW, Wong BH, et al. Severe acute respiratory syndrome coronavirus-like virus in Chinese horseshoe bats. *Proc Natl Acad Sci USA.* (2005) 102:14040–5. doi: 10.1073/pnas.0506735102
43. Li W, Shi Z, Yu M, Ren W, Smith C, Epstein JH, et al. Bats are natural reservoirs of SARS-like coronaviruses. *Science.* (2005) 310:676–9. doi: 10.1126/science.1118391
44. Hu B, Zeng LP, Yang XL, Ge XY, Zhang W, Li B, et al. Discovery of a rich gene pool of bat SARS-related coronaviruses provides new insights into the origin of SARS coronavirus. *PLoS Pathog.* (2017) 13:e1006698. doi: 10.1371/journal.ppat.1006698
45. Wang MN, Zhang W, Gao YT, Hu B, Ge XY, Yang XL, et al. Longitudinal surveillance of SARS-like coronaviruses in bats by quantitative real-time PCR. *Virol Sin.* (2016) 31:78–80. doi: 10.1007/s12250-015-3703-3
46. Cui J, Li F, Shi Z-L. Origin and evolution of pathogenic coronaviruses. *Nat Rev Microbiol.* (2019) 17:181–92. doi: 10.1038/s41579-018-0118-9
47. Raj VS, Farag EA, Reusken CB, Lamers MM, Pas SD, Voermans J, et al. Isolation of MERS coronavirus from a dromedary camel, Qatar, (2014) *Emerg Infect Dis.* (2014) 20:1339–42. doi: 10.3201/eid2008.140663
48. Chu DKW, Hui KPY, Perera R, Miguel E, Niemeyer D, Zhao J, et al. MERS coronaviruses from camels in Africa exhibit region-dependent genetic diversity. *Proc Natl Acad Sci USA.* (2018) 115:3144–9. doi: 10.1073/pnas.1718769115
49. Alagaili AN, Briese T, Mishra N, Kapoor V, Sameroff SC, Burbelo PD, et al. Middle East respiratory syndrome coronavirus infection in dromedary camels in Saudi Arabia. *mBio.* (2014) 5:e00884-14. doi: 10.1128/mBio.01002-14
50. Harcourt JL, Rudoler N, Tamin A, Leshem E, Rasis M, Giladi M, et al. The prevalence of Middle East respiratory syndrome coronavirus (MERS-CoV) antibodies in dromedary camels in Israel. *Zoonoses Public Health.* (2018) 65:749–54. doi: 10.1111/zph.12482
51. Lau SK, Li KS, Tsang AK, Lam CS, Ahmed S, Chen H, et al. Genetic characterization of Betacoronavirus lineage C viruses in bats reveals marked sequence divergence in the spike protein of pipistrellus bat coronavirus HKU5 in Japanese pipistrelle: implications for the origin of the novel Middle East respiratory syndrome coronavirus. *J Virol.* (2013) 87:8638–50. doi: 10.1128/JVI.01055-13
52. Wu F, Zhao S, Yu B, Chen YM, Wang W, Song ZG, et al. A new coronavirus associated with human respiratory disease in China. *Nature.* (2020) 579:265–9. doi: 10.1038/s41586-020-2008-3
53. Wu F, Zhao S, Yu B, Chen YM, Wang W, Song ZG, et al. Author correction: a new coronavirus associated with human respiratory disease in China. *Nature.* (2020) 580:E7. doi: 10.1038/s41586-020-2202-3
54. Zhu N, Zhang D, Wang W, Li X, Yang B, Song J, et al. A novel coronavirus from patients with pneumonia in China, (2019) *N Engl J Med.* (2020) 382:727–33. doi: 10.1056/NEJMoa2001017
55. Zhang YZ, Holmes EC. A genomic perspective on the origin and emergence of SARS-CoV-2. *Cell.* (2020) 181:223–7. doi: 10.1016/j.cell.2020.03.035
56. Coutard B, Valle C, de Lamballerie X, Canard B, Seidah NG, Decroly E. The spike glycoprotein of the new coronavirus 2019-nCoV contains a furin-like cleavage site absent in CoV of the same clade. *Antiviral Res.* (2020) 176:104742. doi: 10.1016/j.antiviral.2020.104742
57. Wrapp D, Wang N, Corbett KS, Goldsmith JA, Hsieh CL, Abiona O, et al. Cryo-EM structure of the 2019-nCoV spike in the prefusion conformation. *Science.* (2020) 367:1260–3. doi: 10.1126/science.abb2507
58. Zhang T, Wu Q, Zhang Z. Probable pangolin origin of SARS-CoV-2 associated with the COVID-19 outbreak. *Curr Biol.* (2020) 30:1346–51.e2. doi: 10.1016/j.cub.2020.03.022
59. Kasibhatla SM, Kinikar M, Limaye S, Kale MM, Kulkarni-Kale U. Understanding evolution of SARS-CoV-2: a perspective from analysis of genetic diversity of RdRp gene. *J Med Virol.* (2020). doi: 10.1002/jmv.25909. [Epub ahead of print].
60. Minskaia E, Hertzig T, Gorbalenya AE, Campanacci V, Cambillau C, Canard B, et al. Discovery of an RNA virus 3'->5' exoribonuclease that is critically involved in coronavirus RNA synthesis. *Proc Natl Acad Sci USA.* (2006) 103:5108–13. doi: 10.1073/pnas.0508200103
61. Agostini ML, Andres EL, Sims AC, Graham RL, Sheahan TP, Lu X, et al. Coronavirus susceptibility to the antiviral remdesivir (GS-5734) is mediated by the viral polymerase and the proofreading exoribonuclease. *mBio.* (2018) 9e00221–18. doi: 10.1128/mBio.00221-18
62. Xiong X, Martin SR, Haire LF, Wharton SA, Daniels RS, Bennett MS, et al. Receptor binding by an H7N9 influenza virus from humans. *Nature.* (2013) 499:496–9. doi: 10.1038/nature12372
63. Skehel JJ, Wiley DC. Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. *Ann Rev Biochem.* (2000) 69:531–69. doi: 10.1146/annurev.biochem.69.1.531
64. Mair CM, Ludwig K, Herrmann A, Sieben C. Receptor binding and pH stability - how influenza A virus hemagglutinin affects host-specific virus infection. *Biochim Biophys Acta.* (2014) 1838:1153–68. doi: 10.1016/j.bbamem.2013.10.004
65. Baldo V, Bertoncello C, Cocchio S, Fonzo M, Pillon P, Buja A, et al. The new pandemic influenza A/(H1N1)pdm09 virus: is it really "new" *J Prev Med Hyg.* (2016) 57:E19–22.
66. Yang J, Li M, Shen X, Liu S. Influenza A virus entry inhibitors targeting the hemagglutinin. *Viruses.* (2013) 5:352–73. doi: 10.3390/v5010352
67. Reiter-Scherer V, Cuellar-Camacho JL, Bhatia S, Haag R, Herrmann A, Lauster D, et al. force spectroscopy shows dynamic binding of influenza hemagglutinin and neuraminidase to sialic acid. *Biophys J.* (2019) 116:1577. doi: 10.1016/j.bpj.2019.03.032
68. Lai JCC, Karunarathna H, Wong HH, Peiris JSM, Nicholls JM. Neuraminidase activity and specificity of influenza A virus are influenced by haemagglutinin-receptor binding. *Emerg Microbes Infect.* (2019) 8:327–38. doi: 10.1080/22221751.2019.1581034
69. Byrd-Leotis L, Cummings RD, Steinhauer DA. The interplay between the host receptor and influenza virus hemagglutinin and neuraminidase. *Int J Mol Sci.* (2017) 18:1541. doi: 10.3390/ijms18071541
70. Li F, Berardi M, Li W, Farzan M, Dormitzer PR, Harrison SC. Conformational states of the severe acute respiratory syndrome coronavirus spike protein ectodomain. *J Virol.* (2006) 80:6794–800. doi: 10.1128/JVI.02744-05
71. Li F, Li W, Farzan M, Harrison SC. Structure of SARS coronavirus spike receptor-binding domain complexed with receptor. *Science.* (2005) 309:1864–8. doi: 10.1126/science.1116480
72. Liu Y, Childs RA, Matrosovich T, Wharton S, Palma AS, Chai W, et al. Altered receptor specificity and cell tropism of D222G hemagglutinin mutants isolated from fatal cases of pandemic A(H1N1) 2009 influenza virus. *J Virol.* (2010) 84:12069–74. doi: 10.1128/JVI.01639-10
73. Wang N, Shi X, Jiang L, Zhang S, Wang D, Tong P, et al. Structure of MERS-CoV spike receptor-binding domain complexed with human receptor DPP4. *Cell Res.* (2013) 23:986–93. doi: 10.1038/cr.2013.92
74. Cao Y, Li L, Feng Z, Wan S, Huang P, Sun X, et al. Comparative genetic analysis of the novel coronavirus (2019-nCoV/SARS-CoV-2) receptor ACE2 in different populations. *Cell Discov.* (2020) 6:11. doi: 10.1038/s41421-020-0147-1
75. Gorbalenya AE, Baker SC, Baric RS, de Groot RJ, Drosten C, Gulyaeva AA, et al. The species severe acute respiratory syndrome-related coronavirus: classifying 2019-nCoV and naming it SARS-CoV-2. *Nat Microbiol.* (2020) 5:536–44. doi: 10.1038/s41564-020-0695-z
76. Huang Q, Herrmann A. Fast assessment of human receptor-binding capability of 2019 novel coronavirus (2019-nCoV). *bioRxiv.* (2020) 2020:2020.02.01.930537. doi: 10.1101/2020.02.01.930537
77. Li M, Li L, Zhang Y, Wang X. *An Investigation of the Expression of 2019 Novel Coronavirus Cell Receptor Gene ACE2 in a Wide Variety of Human Tissues.* Research Square (2020).

78. Gounder AP, Boon ACM. Influenza pathogenesis: the effect of host factors on severity of disease. *J Immunol.* (2019) 202:341–50. doi: 10.4049/jimmunol.1801010
79. Chen IY, Moriyama M, Chang MF, Ichinohe T. Severe acute respiratory syndrome coronavirus viroporin 3a activates the NLRP3 inflammasome. *Front Microbiol.* (2019) 10:50. doi: 10.3389/fmicb.2019.00050
80. Rockx B, Kuiken T, Herfst S, Bestebroer T, Lamers MM, Oude Munnink BB, et al. Comparative pathogenesis of COVID-19, MERS, and SARS in a nonhuman primate model. *Science*. (2020) 2020:eabb7314. doi: 10.1126/science.abb7314
81. Holshue ML, DeBolt C, Lindquist S, Lofy KH, Wiesman J, Bruce H, et al. First case of 2019 novel coronavirus in the United States. *N Engl J Med.* (2020) 382:929–36. doi: 10.1056/NEJMoa2001191
82. Channappanavar R, Perlman S. Pathogenic human coronavirus infections: causes and consequences of cytokine storm and immunopathology. *Semin Immunopathol.* (2017) 39:529–39. doi: 10.1007/s00281-017-0629-x
83. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet*. (2020) 395:497–506. doi: 10.1016/S0140-6736(20)30183-5
84. Zhang W, Zhao Y, Zhang F, Wang Q, Li T, Liu Z, et al. The use of anti-inflammatory drugs in the treatment of people with severe coronavirus disease 2019 (COVID-19): the perspectives of clinical immunologists from China. *Clin Immunol*. (2020) 214:108393. doi: 10.1016/j.clim.2020.108393
85. Tufan A, Avanoglu Guler A, Matucci-Cerinic M. COVID-19, immune system response, hyperinflammation and repurposing antirheumatic drugs. *Turkish J Med Sci.* (2020) 50:620–32. doi: 10.3906/sag-2004-168
86. Xu R, Ekiert DC, Krause JC, Hai R, Crowe JE Jr, et al. Structural basis of preexisting immunity to the 2009 H1N1 pandemic influenza virus. *Science.* (2010) 328:357–60. doi: 10.1126/science.1186430
87. Itoh Y, Shinya K, Kiso M, Watanabe T, Sakoda Y, Hatta M, et al. *In vitro* and *in vivo* characterization of new swine-origin H1N1 influenza viruses. *Nature.* (2009) 460:1021–5. doi: 10.1038/nature08260
88. Schrauwen EJ, de Graaf M, Herfst S, Rimmelzwaan GF, Osterhaus AD, Fouchier RA. Determinants of virulence of influenza A virus. *Eur J Clin Microbiol Infect Dis.* (2014) 33:479–90. doi: 10.1007/s10096-013-1984-8
89. Chen W, Calvo PA, Malide D, Gibbs J, Schubert U, Bacik I, et al. A novel influenza A virus mitochondrial protein that induces cell death. *Nat Med.* (2001) 7:1306–12. doi: 10.1038/nm1201-1306
90. Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y. Evolution and ecology of influenza A viruses. *Microbiol Rev.* (1992) 56:152–79. doi: 10.1128/MMBR.56.1.152-179.1992
91. Moser MR, Bender TR, Margolis HS, Noble GR, Kendal AP, Ritter DG. An outbreak of influenza aboard a commercial airliner. *Am J Epidemiol.* (1979) 110:1–6. doi: 10.1093/oxfordjournals.aje.a112781
92. Verreault D, Moineau S, Duchaine C. Methods for sampling of airborne viruses. *Microbiol Mol Biol Rev.* (2008) 72:413–44. doi: 10.1128/MMBR.00002-08
93. Nicas M, Nazaroff WW, Hubbard A. Toward understanding the risk of secondary airborne infection: emission of respirable pathogens. *J Occup Environ Hyg.* (2005) 2:143–54. doi: 10.1080/15459620590918466
94. Lowen A, Palese P. Transmission of influenza virus in temperate zones is predominantly by aerosol, in the tropics by contact: a hypothesis. *PLoS Curr.* (2009) 1:Rrn1002. doi: 10.1371/currents.RRN1002
95. Polozov IV, Bezrukov L, Gawrisch K, Zimmerberg J. Progressive ordering with decreasing temperature of the phospholipids of influenza virus. *Nat Chem Biol.* (2008) 4:248–55. doi: 10.1038/nchembio.77
96. Otter JA, Yezli S, French GL. The role played by contaminated surfaces in the transmission of nosocomial pathogens. *Infect Control Hosp Epidemiol.* (2011) 32:687–99. doi: 10.1086/660363
97. Bean B, Moore BM, Sterner B, Peterson LR, Gerding DN, Balfour HH Jr. Survival of influenza viruses on environmental surfaces. *J Infect Dis.* (1982) 146:47–51. doi: 10.1093/infdis/146.1.47
98. van Doremalen N, Bushmaker T, Munster VJ. Stability of Middle East respiratory syndrome coronavirus (MERS-CoV) under different environmental conditions. *Euro Surveill.* (2013) 18:20590. doi: 10.2807/1560-7917.ES2013.18.38.20590
99. Duan SM, Zhao XS, Wen RF, Huang JJ, Pi GH, Zhang SX, et al. Stability of SARS coronavirus in human specimens and environment and its sensitivity to heating and UV irradiation. *Biomed Environ Sci.* (2003) 16:246–55.
100. Chan KH, Peiris JS, Lam SY, Poon LL, Yuen KY, Seto WH. The effects of temperature and relative humidity on the viability of the SARS coronavirus. *Adv Virol.* (2011) 2011:734690. doi: 10.1155/2011/734690
101. van Doremalen N, Bushmaker T, Morris DH, Holbrook MG, Gamble A, Williamson BN, et al. Aerosol and surface stability of SARS-CoV-2 as compared with SARS-CoV-1. *N Engl J Med.* (2020) 382:1564–7. doi: 10.1056/NEJMc2004973
102. Wearing HJ, Rohani P, Keeling MJ. Appropriate models for the management of infectious diseases. *PLoS Med*. (2005) 2:e174. doi: 10.1371/journal.pmed.0020174
103. Heffernan JM, Smith RJ, Wahl LM. Perspectives on the basic reproductive ratio. *J R Soc Interface*. (2005) 2:281–93. doi: 10.1098/rsif.2005.0042
104. Biggerstaff M, Cauchemez S, Reed C, Gambhir M, Finelli L. Estimates of the reproduction number for seasonal, pandemic, and zoonotic influenza: a systematic review of the literature. *BMC Infect Dis.* (2014) 14:480. doi: 10.1186/1471-2334-14-480
105. Chowell G, Castillo-Chavez C, Fenimore PW, Kribs-Zaleta CM, Arriola L, Hyman JM. Model parameters and outbreak control for SARS. *Emerg Infect Dis.* (2004) 10:1258–63. doi: 10.3201/eid1007.030647
106. Breban R, Riou J, Fontanet A. Interhuman transmissibility of Middle East respiratory syndrome coronavirus: estimation of pandemic risk. *Lancet*. (2013) 382:694–9. doi: 10.1016/S0140-6736(13)61492-0
107. D'Arienzo M, Coniglio A. Assessment of the SARS-CoV-2 basic reproduction number, R0, based on the early phase of COVID-19 outbreak in Italy. *Biosaf Health*. (2020) 2:57–9. doi: 10.1016/j.bsheal.2020.03.004
108. Fineberg HV. Pandemic preparedness and response — lessons from the H1N1 influenza of 2009. *N Engl J Med*. (2014) 370:1335–42. doi: 10.1056/NEJMra1208802
109. Russell CA, Kasson PM, Donis RO, Riley S, Dunbar J, Rambaut A, et al. Science Forum: improving pandemic influenza risk assessment. *eLife*. (2014) 3:e03883. doi: 10.7554/eLife.03883
110. WHO. *Pandemic Influenza Risk Management (PIRM)*. (2017). Available online at: https://www.who.int/influenza/preparedness/pandemic/influenza_risk_management/en/
111. Prem K, Liu Y, Russell TW, Kucharski AJ, Eggo RM, Davies N, et al. The effect of control strategies to reduce social mixing on outcomes of the COVID-19 epidemic in Wuhan, China: a modelling study. *Lancet Public Health*. (2020) 5:e261–70. doi: 10.1101/2020.03.09.20033050
112. McDonald LC, Simor AE, Su IJ, Maloney S, Ofner M, Chen KT, et al. SARS in healthcare facilities, Toronto and Taiwan. *Emerg Infect Dis.* (2004) 10:777–81. doi: 10.3201/eid1005.030791
113. Suwantarat N, Apisarnthanarak A. Risks to healthcare workers with emerging diseases: lessons from MERS-CoV, Ebola, SARS, and avian flu. *Curr Opin Infect Dis.* (2015) 28:349–61. doi: 10.1097/QCO.0000000000000183
114. WHO. *Emergencies Preparedness, Response*. (2004). Available online at: https://www.who.int/csr/resources/publications/WHO_CDS_CSR_ARO_2004_2/en/
115. Memish ZA, Cotten M, Meyer B, Watson SJ, Alsahafi AJ, Al Rabeeah AA, et al. Human infection with MERS coronavirus after exposure to infected camels, Saudi Arabia, 2013. *Emerg Infect Dis*. (2014) 20:1012. doi: 10.3201/eid2006.140402
116. Peeri NC, Shrestha N, Rahman MS, Zaki R, Tan Z, Bibi S, et al. The SARS, MERS and novel coronavirus (COVID-19) epidemics, the newest and biggest global health threats: what lessons have we learned? *Int J Epidemiol*. (2020) 49:717–26. doi: 10.1093/ije/dyaa033
117. Devaux CA, Rolain JM, Colson P, Raoult D. New insights on the antiviral effects of chloroquine against coronavirus: what to expect for COVID-19? *Int J Antimicrob Agents*. (2020) 2020:105938. doi: 10.1016/j.ijantimicag.2020.105938
118. Cortegiani A, Ingoglia G, Ippolito M, Giarratano A, Einav S. A systematic review on the efficacy and safety of chloroquine for the treatment of COVID-19. *J Crit Care*. (2020) 57:279–83. doi: 10.1016/j.jcrc.2020.03.005
119. Savarino A, Boelaert JR, Cassone A, Majori G, Cauda R. Effects of chloroquine on viral infections: an old drug against today's diseases? *Lancet Infect Dis.* (2003) 3:722–7. doi: 10.1016/S1473-3099(03)00806-5

120. Huang Z, Liu H, Zhang X, Wen G, Zhu C, Zhao Y, et al. Transcriptomic analysis of lung tissues after hUC-MSCs and FTY720 treatment of lipopolysaccharide-induced acute lung injury in mouse models. *Int Immunopharmacol.* (2018) 63:26–34. doi: 10.1016/j.intimp.2018.06.036
121. Zhang Z, Li W, Heng Z, Zheng J, Li P, Yuan X, et al. Combination therapy of human umbilical cord mesenchymal stem cells and FTY720 attenuates acute lung injury induced by lipopolysaccharide in a murine model. *Oncotarget*. (2017) 8:77407–14. doi: 10.18632/oncotarget.20491
122. Wu W, Wang JF, Liu PM, Chen WX, Yin SM, Jiang SP, et al. [Clinical features of 96 patients with severe acute respiratory syndrome from a hospital outbreak]. *Zhonghua Nei Ke Za Zhi.* (2003) 42:453–7.
123. Stockman LJ, Bellamy R, Garner PJ. SARS: systematic review of treatment effects. *PLoS Med*. (2006) 3:e343. doi: 10.1371/journal.pmed.0030343
124. Sharif-Yakan A, Kanj SS. Emergence of MERS-CoV in the Middle East: origins, transmission, treatment, and perspectives. *PLoS Pathog*. (2014) 10:e1004457. doi: 10.1371/journal.ppat.1004457
125. Jefferson T, Jones M, Doshi P, Spencer EA, Onakpoya I, Heneghan CJ. Oseltamivir for influenza in adults and children: systematic review of clinical study reports and summary of regulatory comments. *BMJ*. (2014) 348:g2545. doi: 10.1136/bmj.g2545
126. Peramivir for influenza. *Aust Prescr*. (2019) 42:143. doi: 10.18773/austprescr.2019.047
127. Cheng VC, Wong S-C, To KK, Ho P, Yuen K-Y. Preparedness and proactive infection control measures against the emerging novel coronavirus in China. *J Hosp Infect*. (2020) 104:254–5. doi: 10.1016/j.jhin.2020.01.010
128. Chartier Y, Pessoa-Silva C. *Natural Ventilation for Infection Control in Health-Care Settings*. World Health Organization (2009).

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Public Health

MINI REVIEW
published: 11 September 2020
doi: 10.3389/fpubh.2020.555550

The Epidemiology of COVID-19 in the Gansu and Jinlin Provinces, China

Jingchun Fan[1,2], *Brett D. Hambly*[1,3] *and Shisan Bao*[1,3]*

[1] School of Public Health, Gansu University of Chinese Medicine, Lanzhou, China, [2] Center for Evidence-Based Medicine, Gansu University of Chinese Medicine, Lanzhou, China, [3] Discipline of Pathology, Faculty of Medicine and Health, The School of Medical Sciences, The University of Sydney, Sydney, NSW, Australia

The COVID-19 outbreak has become a pandemic. The outbreak was able to be controlled in China by mid-April through the implementation of critical measures; however, significant reverse transmission has resulted in hot spots perturbing prevention and control. To date, there have only been a total of 92 indigenous COVID-19 cases confirmed in the Gansu Province, which is considered to be a consequence of the strict screening approach applied during the outbreak. The emergency response level to COVID-19 were able to be decreased from high to low, despite some relatively minor reverse transmission cases from other countries in March 2020. The stringent preparative measures undertaken by the Gansu authorities, involving high-level, streamlined cooperation between the transportation, quarantine, and medical resource departments, have underpinned this success. There has been an emergence of clusters of freshly infected COVID-19 patients in the Jilin Province in northeast China. The single largest cluster has been in Shulan of the Jilin Province, involving 43 confirmed infections. A strict lockdown was implemented immediately. The source of the current outbreak of COVID-19 is suggested to be travelers returning from Russia. The current strategy from the Chinese authorities is aimed at preventing reverse transmission via international importation to avert a rebound of COVID-19 in China. These data highlight the need for an exceptionally high level of vigilance and for a pre-emptive response that is informative for the development of policy to prevent a second and further waves of infections in general.

Keywords: SARS-CoV-2, China, COVID-19, second wave, primary wave

OPEN ACCESS

Edited by:
Zisis Kozlakidis, International Agency For Research On Cancer (IARC), France

Reviewed by:
Martina Barchitta, University of Catania, Italy
Ghobad Moradi, Kurdistan University of Medical Sciences, Iran

***Correspondence:**
Shisan Bao
bob.bao@sydney.edu.au

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Public Health

Received: *25 April 2020*
Accepted: *14 August 2020*
Published: *11 September 2020*

Citation:
Fan J, Hambly BD and Bao S (2020) The Epidemiology of COVID-19 in the Gansu and Jinlin Provinces, China. Front. Public Health 8:555550. doi: 10.3389/fpubh.2020.555550

INTRODUCTION

2019 novel coronavirus disease (COVID-19), caused by infection with SARS-CoV-2 virus, was originally discovered in Wuhan, Hubei Province of Central China in December 2019 (1). The outbreak of COVID-19 was far more severe than anyone expected due to insufficient knowledge of the SARS-CoV-2 virus transmission during the initial stages of the spread (2). Currently (as at August 12, 2020), data on the extent of the pandemic are as follows: the pandemic has involved 215 countries and territories with a total of 19,936,210 confirmed cases that have been reported, including a total of 732,499 deaths (3). Wuhan is located in Central China with a population of 15 million (4). Due to the impending Chinese New Year, more than 5 million people traveled from Wuhan for either family reunions and/or holidays (5), contributing to the subsequent outbreak of COVID-19 in every province/region in China within a matter of weeks (6) that evolved into a pandemic within a matter of months (7). In response to the spread of the virus, a strict lockdown was implemented in late January 2020 in China in an attempt to stop person-to-person

transmission, including the mandatory use of face masks in public, no public gatherings, and school and factory closures (8). It has been striking to observe that these measures were able to substantially reduce the number of COVID-19 cases to close to zero within a month, i.e., by February 2020 (9). In addition, mandatory COVID-19 testing was instigated for all staff and patients in every in-patient department in all hospitals, including accompanying family members (10). As expected, subsequently there have been almost no new COVID-19 cases reported in China (11). Since late April 2020, almost all schools in China have been allowed to re-open, following initial online teaching only during March and April 2020 (12). Manufacturing industries around the country have gradually reopened following the reopening of schools (13). This evidence supports the remarkable achievement in controlling the outbreak of COVID-19 within China (14).

While most publications by clinicians and researchers have been focusing on the epicenter of COVID-19, i.e., Wuhan, China, this manuscript aims to cover the epidemiology of the COVID-19 infection in the Northern region of China, namely the Gansu (Northwest) and Jilin (Northeast) Provinces. The primary outbreaks in the Gansu and Jilin Provinces were very similar and mild during the first wave that occurred in January and February 2020. However, once the initial outbreak was under control, Gansu accepted the task of quarantining Chinese nationals returning from abroad and undertook to provide treatment for those returnees who were infected with coronavirus. On the other hand, Jilin subsequently experienced a second wave of infection triggered by asymptomatic cases. In this review, we will outline the differences in the epidemiological approaches adopted by the two provinces in northern China to provide the scientific basis for epidemic prevention and control.

IMPORTANCE OF THE PUBLIC HEALTH RESPONSE

We hypothesize that the general population continues to face dangerous SARS-CoV-2 viral transmission from distant locations, including from the epicenter (Wuhan, Hubei Province, China), if no effective measures are implemented, despite considerable precautions being undertaken by the provincial governmental authorities. One of the current critical challenges in China is to detect and avert possible reverse transmission of SARS-CoV-2 virus from overseas. The information from our current studies provides some key points that could be used by other regions/countries where COVID-19 is still not yet over the peak of the outbreak.

Recently, we have demonstrated that the primary COVID-19 cases seen in northern China were originally transmitted from Wuhan, Hubei Province, China (2). It is well-documented that COVID-19, originally discovered in Wuhan in late December 2019, was transmitted to Northern China (2). We have reported that within 10 days, from January 23 to February 3, 2020, there were 54 people infected with the SARS-CoV-2 virus, where 35 cases had traveled from Wuhan, and 19 were infected by close contact with the identified travelers. However, the identification of case zero or the index case in northern China could not be made with absolute certainty because the index case in most countries has been found to be asymptomatic (15). Thus, it is critically important to develop novel diagnostic tool(s) with both high sensitivity and specificity to combat this devastating pandemic. Our data suggest that the implementation of adequate interventions has been able to decrease transmission of the COVID-19 virus in the Gansu Province. Following the pandemic of COVID-19 within months of the original outbreak in China, the countries most affected at the time point of March 2020 were Italy (16) and Iran (17). Despite some precautions being undertaken in Italy and Iran in late February 2020, e.g., reducing public gathering and implementing social distancing in Italy and cancellation of mosque worship in Iran and blockage of interstate travel (18), the morbidity and mortality was still able to increase with enormous speed in early March 2020 (19). The increased incidence of COVID-19 in Italy and Iran after emergency response measures were implemented may be due to the long incubation period of the SARS-CoV-2 viral infection, which may be up to 20 days (20). Furthermore, this rapid spread may also be due to relatively low adherence to the restriction orders within these two countries (21). To provide shelter for the overseas Chinese residents in risky countries from the potential risk of COVID-19, the Chinese government provided chartered planes to repatriate these Chinese citizens back to China (22). The destination for these returnees from Italy and Iran was the Zhejiang and Gansu Provinces, respectively (23).

PRIMARY OUTBREAK OF COVID-19 IN THE JILIN AND GANSU PROVINCES, CHINA

We have reviewed the epidemiology of COVID-19 in Jilin and Gansu provinces, Northern China. Jilin Province is located in the middle of the northeast of China, covering an area of 187,400 km^2 with a total population of 27,746,000. Due to the strategically important location in the northeast of China, the Jilin Province is an important gateway connecting the Eurasian land route via Siberia, e.g., the Jilin Province is only 4 km from Vladivostok, Russia, and 15 km from the Sea of Japan (24). On the other hand, the Gansu Province is very similar to the Jilin Province in several aspects. The Gansu Province is located in the northwest of China, covering an area of 454,000 km^2 with a total population of 26,257,100 (25). Geographically the Gansu Province is also a key transportation hub connecting to five provinces in northwest China. Although the Gansu Province is located in a rather remote region in northwest China, it is considered to be the beginning of the Silk Road (**Figure 1**). During the primary outbreak, there were 93 and 92 cases, including two deaths, in the Jilin and Gansu Provinces, respectively (26, 27).

SECOND WAVE: REVERSE TRANSMISSION OF COVID-19

The epidemic of COVID-19 was brought under control in these two provinces in March 2020 and remained under control for

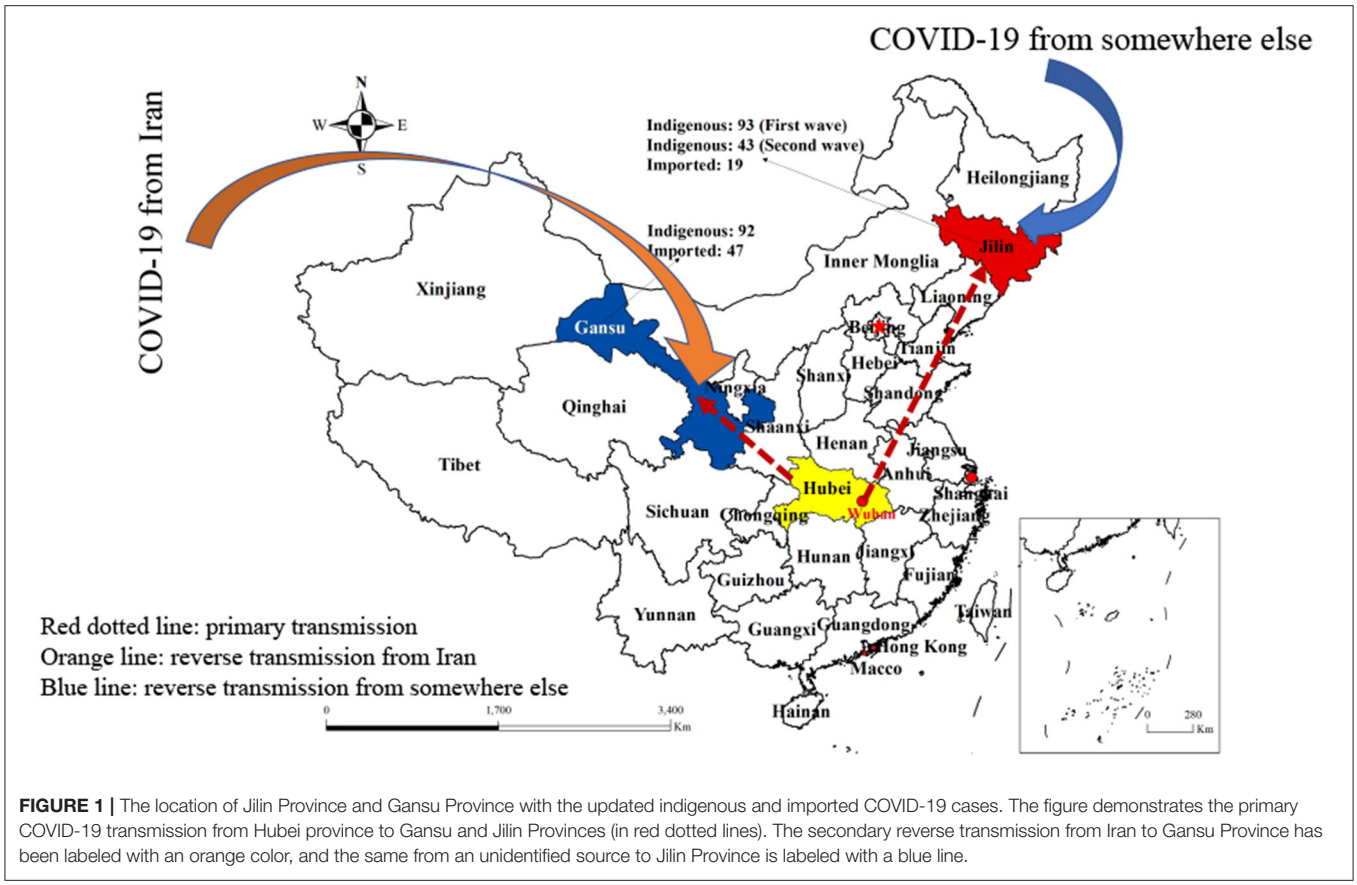


FIGURE 1 | The location of Jilin Province and Gansu Province with the updated indigenous and imported COVID-19 cases. The figure demonstrates the primary COVID-19 transmission from Hubei province to Gansu and Jilin Provinces (in red dotted lines). The secondary reverse transmission from Iran to Gansu Province has been labeled with an orange color, and the same from an unidentified source to Jilin Province is labeled with a blue line.

almost 2 months. In response to this level of control, the Jilin government proposed a series of measures to control COVID-19. Similarly, within the Gansu Province, the schools have reopened, and the enterprises have been able to recover and re-commence production. During May 2020, however, there has been a re-emergence of COVID-19 in northeast China, particularly in the Jilin Province, while fortunately northwest China has remained free of new infections. It has been reported from the Reuters news agency that another outbreak of COVID-19 has been detected in the Jilin Province, in northeast China, since the removal of the restrictive lockdown and the increase in public activity since April 8, 2020 (28).

A single new case of COVID-19 was discovered on May 7, 2020, in Shulan, in the Jilin Province, without any obvious history of contact with COVID-19 patients and also without a history of interstate/international travel. Additionally, 11 COVID-19 cases were confirmed on May 9, 2020, which broke the record of 73 days of no new cases in this city (26). An immediate lockdown was then implemented in Shulan, Jilin, from May 10, 2020, and the risk level was increased from level II (moderate) to level III (high) (26). The prevention and control measures implemented in Shulan are as high as those in Wuhan at the peak of infection, which was the original epicenter of COVID-19. However, despite the strict lockdown in Shulan, the number of new COVID-19 cases has continued to increase to 43 as of May 20, 2020, which is thought to be a consequence of the close contact of the infected people in this clustering outbreak. It has been reported that the Chinese national returnees coming from Russia have mainly traveled back via train, suggesting that poor screening and quarantine measures may have occurred, and that most imported cases in the Jilin Province are from Russia (26).

In response to the primary COVID-19 outbreak in their Provinces, the authorities from both the Gansu and Jilin Provinces found themselves dealing with this form of emergency for the first time (2). The response included strict prohibition of public gatherings, limitation of social activity to an extremely minimal level, and in-house working via the internet during the outbreak of COVID-19 (29, 30) and these measures were rigorously adhered to. These emergency approaches to deal with COVID-19 were closely modeled on those developed within the epicenter, Wuhan, Hubei Province of China. However, during the reverse transmission outbreak (or second wave), these provincial authorities had already had previous experience in dealing with this subsequent COVID-19 outbreak (22).

Infection in the Gansu Province has been shown to have occurred in two stages. The first stage was the imported case stage of the epidemic, meaning that the cases arrived in travelers from Wuhan (2). In the second indigenous case stage, the patients have been mainly shown to have been infected by the cases from the first imported stage (2). Importantly, during the progression of the COVID-19 epidemic in the Gansu Province, the basic reproduction number (R_0) has been shown to have decreased

from 2.61 in the first stage to 0.66 in the second stage (31), which largely due to the substantially more strict social distancing arrangements implemented during the second stage. New cases of COVID-19 almost reached zero in Northern China within the period from late March 2020 to the middle of May 2020 because of the implementation of restrictive orders. In addition, the clinical interventions for COVID-19 patients were also effective and efficient in reducing morbidity and mortality, in addition to the restrictive quarantine approach (32). Consequently, the mortality rate in Northern China was only two in the Gansu (26) and Jilin Provinces (27).

As the epidemic progressively came under control in China, an alarmingly rapid spread of the virus occurred worldwide, and the epidemic became a pandemic. As the preferred place for receiving Chinese returnees by the Chinese authority, Lanzhou city has received a total of 311 evacuated Chinese citizens from Iran, amongst whom there has been 37 confirmed positive cases of COVID-19 infection, which were only discovered shortly after arrival in Lanzhou (32). Compared to the handling procedures utilized during the primary outbreak of COVID-19 in the Gansu province, the local government had gained substantially increased knowledge and experience in controlling the transmission of SARS-CoV-2 virus that they were able to apply during the secondary reverse transmission of COVID-19 (32). Consequently, due to a substantially more organized level of preparation, local Gansu authorities were able to implement an effective approach in advance of the evacuation, involving high-level, streamlined cooperation among the departments of transportation, quarantine and hospitals, aiming to isolate, and quarantine for 14 days all potentially infected evacuees within designated hotels to prevent the potential risk of transmission of SARS-CoV-2 virus within the Chinese communities of origin of the evacuees. In addition to these organized returnees from Iran, 10 COVID-19 patients have been confirmed in the Gansu Province among independent travelers from abroad who have traveled from locations such as Saudi Arabia and the United States of America (33).

Unfortunately, a proportion of these infected international travelers who returned to China, including to the Jilin and Gansu Provinces, during the early stages of international spread before March 2020 were able to scatter within the community without being quarantined (34), which caused a significant potential risk of the spread of COVID-19. The reason why these COVID-19-infected travelers were able to scatter within their local provinces was that no testing for COVID-19 was undertaken, as COVID-19 testing for returnees was not mandatory in early March 2020—the beginning of the first wave of the outbreak. Subsequently, the local authorities have learnt a heavy lesson from these mistakes and implemented much greater restrictive orders. These data highlight the need for an exceptionally high level of vigilance and the need for a pre-emptive response to prevent a second wave occurring within a community, where the pandemic had been successfully controlled, from returnees from other international locations where the extent of infection at those distant sites had not yet been fully realized.

With the recognition of the seriousness of the SARS-CoV-2 virus in May 2020, the local and central governments called for strengthening of border biosecurity controls, including in the North-eastern provinces, e.g., the Jilin Province, where a growing cluster of infections near the Russian and North Korean borders has threatened to develop into a second wave (35). In addition to the lockdown in the Jilin Province, in order to further reduce possible inadvertent transmission, all private clinics in the Jilin Province have been temporarily suspended until further notice. All patients requiring assessment are now required to attend public hospitals for help, especially for any patients with suspected symptoms associated with COVID-19 who should go to the specialist fever clinics. Thus, Chinese authorities have sought to exhibit flexibility with a rapid response time to enhance the control of the COVID-19 epidemic in key areas that require increasing regular prevention measures in line with the changing situation of the outbreak (36).

One effective approach that has been applied by the Chinese authorities is to launch a health QR (quick response) code system on each individual's smartphone; it is intended to offer a reasonably good indicator within the general population of potential infective status to keep the virus from spreading further. The healthy tracking application has been used previously in monitoring other chronic illnesses for several different purposes (37). This healthy tracking system provides either a green or red code, i.e., non-infected or infected person, respectively. This rating system permits the green code individuals to restart normal activities with minimal risk of infection to others. However, the health QR code system is not foolproof. For example, there has been one individual in Lanzhou with a green code who had traveled from the Hubei Province. A nasal swab RNA test later confirmed that this individual was infected with COVID-19 but asymptomatic (38).

It should be cautioned that there is no significant difference in the secondary infection rate of COVID-19 within the population, caused by infected individuals who are either symptomatic or asymptomatic (39). With this in mind, the Chinese authorities have also been paying particular attention to the detection of asymptomatic cases to prevent further spreading. Interestingly, a comparable project, the Australian Sentinel Practice Research Network (ASPREN) surveillance program, is currently being used for COVID-19 detection in Australia, which was originally intended for monitoring influenza-like illnesses (40). Nevertheless, this approach is in line with an Australian proposal of a system of sentinel testing of people in which large numbers of random, but potentially risky, individuals have been presumptively tested irrespective of showing any symptoms. Such an approach has enabled the authorities to gauge the extent of asymptomatic carriers and detect infection clusters before any infected individuals develop clinical symptoms (41).

Thus, it is essential for the authorities in China to identify these potential COVID-19 risk populations, including local residents and/or overseas returnees, using a more sensitive diagnostic approach, e.g., detection of serum antibodies (42), in addition to nucleic acid testing, which only detects the presence of the virus. Such an approach probably offers greater reliability and flexibility in dealing with potentially infected people within an infection cluster.

SCREENING POLICY AND CONTROLLING STRATEGIES

In Wuhan, the epicenter of COVID-19 infection, a series of policies were implemented. It was confirmed that SARS-CoV-2 virus was able to be transmitted from person-to-person on January 20, 2020. Although COVID-19 was classified as a category B infectious disease, the procedures for preventing and controlling category A infectious diseases (e.g., plague and cholera) were adopted (43). The implementation of these procedures was undertaken by the Wuhan local government, including, firstly, the mandatory wearing of facial masks in a list of public places, including hotels and department stores, and, secondly, strict limitations on outdoor and group activities, particularly in relation to banning public and/or private social gatherings (44). Finally, a complete lockdown of Wuhan was commenced on January 23, 2020, and it lasted for 76 days until April 8, 2020 (45), including a complete shutdown of manufacturing facilities and shops except for essential food and groceries.

Following the concurrent confirmation of the first COVID-19 case on January 23, 2020, in the Gansu and Jilin Provinces (1, 46), the Gansu and Jilin provincial governments immediately implemented the following policies for preventing and controlling COVID-19; emergency response measures were raised to the highest level, effective immediately, which was equivalent to the policies applied in Wuhan at the same time. The emergency response levels to any infectious diseases are classified by the National Health Commission of China (47).

General population screening in China has included mandatory temperature checking for everyone entering any building, using a temperature gun. In addition, for quarantine purposes, monitoring of people's movements was undertaken using a smartphone QR health code system, where an on-screen QR code (for a quick response) was required at the entrance to all buildings to facilitate contact tracing in the event that any positive case was confirmed within the building. Any person with a continuous abnormal temperature was required to have a COVID-19 RNA test for screening confirmation (47). Nevertheless, using this high-level emergency response has proven to be extremely useful, demonstrating that COVID-19 has been effectively brought under control. Consequently, on February 26 and March 2, 2020, the Jilin and Gansu governments lowered the emergency response measures from high to medium and high to low, respectively (48, 49). However, the policy of screening within the general population, i.e., temperature monitoring, and the use of the QR code app are still being used as a major screening approach to the present time (June 2020).

In northern China, the sequential procedures that were adopted were as follows: city lockdown, use of road blocks except for essential travel, maintenance of social distancing, restrictions on social gatherings, mandatory wearing of face masks in public, closure of manufacturing facilities and schools, temperature checking at building entrances, reporting of whereabouts and health condition via QR code app, and remote online working and schooling in the Gansu and Jilin Provinces during the first wave of COVID-19. In response to the second wave in the Jilin Province, the emergency response was immediately re-implemented as described above. The series of strategies implemented to control COVID-19 spreading in Wuhan were essentially the same procedures that were utilized in both the Gansu and Jilin Provinces, the only difference being the commencement and finishing times (**Table 1**).

TABLE 1 | The strategies implemented to control COVID-19 epidemic spread (taking Wuhan as an example).

No.	Strategies	Implementation duration
1	Keep social distance	20 January to 8 April 2020
2	Restrict social gathering	20 January to 8 April 2020
3	Mandatory face mask wearing in public	22 January 2020 till now
4	Suspending production	22 January to 8 April 2020
5	School closure	22 January 2020 till now
6	Temperature checking at building entrance	23 January 2020 till now
7	Lockdown of Wuhan	23 January to 8 April 2020
8	Blockage of unnecessary traveling	23 January to 8 April 2020
9	QR code reporting health condition	23 January to 8 April 2020
10	Internet remote working and schooling	10 February 2020 till now

CONCLUSION

In conclusion, COVID-19 is almost completely controlled in the general population of the Gansu Province in northwest China. The first lesson we have learnt from these studies, up to the present time, is that the SARS-CoV-2 virus is able to be transmitted among people very effectively. Thus, it is necessary that strict prohibition of public gatherings, limiting social activity to an extremely minimal level, and remote online working during the outbreak of COVID-19 (29, 30) should be rigorously adhered to. However, it is still debatable whether mandatory wearing of face masks should be undertaken (50, 51). From a public health and safety point of view, it is crucial to continue robust vigilance and implement aggressive control measures to prevent further outbreaks of COVID-19 until complete containment of the pandemic is achieved.

LIMITATIONS

Despite using primary data collected from the local health officials (23), we acknowledge that there are limitations for the current mini review. One limitation is that there has been only one original research paper published concerning the recent and current situation in the Jilin Province (46). Additionally, our available data are insufficient to calculate the R_0 in the Jilin Province and the R_0 during the second wave in the Gansu Province, and there has been no published data concerning the R_0 in these two provinces, which we will determine in our future studies. Notably, the most significant outcomes of the second wave of the outbreak in the Jilin Province are still evolving and hence are not settled yet. This is despite all necessary measures

that were used in the control of the first wave of COVID-19 having been implemented.

AUTHOR CONTRIBUTIONS

JF and SB have concieved the manuscript and wrote it. BH has editted the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by the Talent Introduction Program of Gansu University of Chinese Medicine (no. 2016YJRC-01), The Special project on COVID-19 Emergency (JF), Prevention and Treatment of Gansu University of Chinese Medicine 2020XGZX-10 (JF), and SJTU grants from The University of Sydney (SB).

REFERENCES

1. Zhu N, Zhang D, Wang W, Li X, Yang B, Song J, et al. A novel coronavirus from patients with pneumonia in China, 2019. *N Engl J Med*. (2020) 382:727–33. doi: 10.1056/NEJMoa2001017
2. Fan J, Liu X, Pan W, Douglas M, Bao S. Epidemiology of 2019 novel coronavirus disease-19 in Gansu Province, China, 2020. *Emerg Infect Dis*. (2020) 26:1257–65. doi: 10.3201/eid2606.200251
3. World Health Organization. *Coronavirus Disease (COVID-19) Situation Report-204*. (2020). Available online at: https://www.who.int/docs/default-source/coronaviruse/situation-reports/20200811-covid-19-sitrep-204.pdf?sfvrsn=1f4383dd_2 (accessed August 11, 2020).
4. Hubei Provincial Bureau of Statistics. *Hubei Statistical Yearbook-2018*. Wuhan: Hubei Provincial Bureau of Statistics (2019).
5. Ma J, WND News Services. *5 Million Left Wuhan Before Lockdown, 1,000 New Coronavirus Cases Expected in City*. (2020). Available online at: https://ph.news.yahoo.com/5-million-residents-left-wuhan-142328311.html (accessed January 22, 2020).
6. National Health Commission of the People's Republic of China. *Update on Novel Coronavirus Infection at 24 Hours on 29 January*. (2020). Available online at: http://www.nhc.gov.cn/xcs/yqtb/202001/e71bd2e7a0824ca69f87bbf1bef2a3c9.html (accessed January 30, 2020).
7. World Health Organization. *Coronavirus Disease 2019 (COVID-19) Situation Report-100*. (2020). Available online at: https://www.who.int/docs/default-source/coronaviruse/situation-reports/20200425-sitrep-100-covid-19.pdf?sfvrsn=a33836bb_2 (accessed May 1, 2020).
8. Fan J, Gao Y, Zhao N, Dai R, Zhang H, Feng X, et al. Bibliometric analysis on COVID-19: a comparison of research between english and Chinese studies. *Front Public Health*. (2020) 8:477. doi: 10.3389/fpubh.2020.00477
9. Health Commission of Gansu Province. *Change of Risk of New Crown Pneumonia in Sub-Counties of Gansu Province*. (2020). Available online at: http://wsjk.gansu.gov.cn/single/10910/84554.html (accessed March 2, 2020).
10. National Health Commission of People's Republic of China. *Notice on Further Expediting the Novel Coronavirus Nucleic Acid Detection Capability in Medical Institutions*. (2020). Available online at: http://www.nhc.gov.cn/xcs/zhengcwj/202007/5b5597bcbffc4623b0b9ec051a0f173d.html (accessed June 2, 2020).
11. Guo S, Zhang W, Ni W. Strategies for front-line medical staff to prevent occupational exposure Infection to novel coronavirus. *J Chongqing Med Univ*. (2020). doi: 10.13406/j.cnki.cyxb.002552
12. Bao W. COVID-19 and online teaching in higher education: a case study of Peking University. *Hum Behav Emerg Technol*. (2020) 2:113–5. doi: 10.1002/hbe2.191
13. Ministry of Education of People's Republic of China. *Scientific and Precise Epidemic Prevention and Control will Promote the Resumption of all Schools and Classes*. (2020). Available online at: http://www.moe.gov.cn/jyb_xwfb/s5147/202005/t20200514_454040.html (accessed May 14, 2020).
14. National Health Commission of the People's Republic of China. *Situation Report-Update on COVID-19 up to March 31, 2020*. (2020). Available online at: http://www.nhc.gov.cn/xcs/yqfkdt/202003/e644c2fc18b4448db7ed4b30f68b91a6.html (accessed April 1, 2020).
15. Zhang W, Cheng W, Luo L, Ma Y, Xu C, Qin P, et al. Secondary transmission of coronavirus disease from presymptomatic person, China. *Emerg Infect Dis*. (2020) 26:1924–6. doi: 10.3201/eid2608.201142
16. Giovanetti M, Benvenuto D, Angeletti S, Ciccozzi M. The first two cases of 2019-nCoV in Italy: where they come from? *J Med Virol*. (2020) 92:518–21. doi: 10.1002/jmv.25699
17. World Health Organization. *Coronavirus Disease 2019 (COVID-19) Situation Report—63*. (2020). Available online at: https://www.who.int/docs/default-source/coronaviruse/situation-reports/20200302-sitrep-63-covid-19.pdf?sfvrsn=224c1add_2 (accessed March 23, 2020).
18. TravelWire News. *COVID-19: Coronavirus Changing Way Muslims Across World Worship*. (2020). Available online at: https://travelwirenews.com/covid-19-coronavirus-changing-way-muslims-across-world-worship-1684670/ (accessed March 4, 2020).
19. Centres for Disease Control and Prevention. *A Weekly Surveillance Summary of U.S. COVID-19 Activity*. (2020). Available online at: https://www.cdc.gov/coronavirus/2019-ncov/covid-data/covidview/index.html (accessed June 5, 2020).
20. Arashiro T, Furukawa K, Nakamura A. COVID-19 in 2 persons with mild upper respiratory symptoms on a cruise ship, Japan. *Emerg Infect Dis*. (2020) 26:1345–8. doi: 10.3201/eid2606.200452
21. Signorelli C, Scognamiglio T, Odone A. COVID-19 in Italy: impact of containment measures and prevalence estimates of infection in the general population. *Acta Biomed*. (2020) 91:175–9. doi: 10.23750/abm.v91i3-S.9511
22. Air China. *Why Would an Iranian Charter Plane Fly to Lanzhou Directly?* (2020). Available online at: http://www.aviation.cn/2020/0306/65351.html (accessed March 6, 2020).
23. Health Commission of Jilin Province. *Notification on Adjusting the Classification of COVID-19 Epidemic Prevention and Control in Jilin Province*. (2020). Available online at: http://www.jl.gov.cn/zw/yw/jlyw/202005/t20200510_7205373.html (accessed May 10, 2020).
24. The People's Government of Jilin. *Overview of Jilin Province*. (2019). Available online at: http://english.jl.gov.cn/ABOUTJILIN/JILINFACTS/201911/t20191128_6191801.html (accessed November 28, 2019).
25. Gansu Provincial Bureau of Statistics. *Gansu Statistical Yearbook-2018–2019*. Lanzhou: Gansu Provincial Bureau of Statistics (2020).
26. The People's Government of Jilin. *Epidemic Situation and Trend Chart of Jilin Province*. (2020). Available online at: http://www.jl.gov.cn/szfzt/jlzxd/yqtb/202005/t20200504_7194214.html (accessed May 4, 2020).
27. Health Commission of Gansu Province. *COVID-19 Situation Report in Gansu Province*. (2020). Available online at: http://wsjk.gansu.gov.cn/single/10910/85810.html (accessed May 19, 2020).
28. The Reuters. *China's Jilin City Warns of Huge Risk of Further COVID-19*. (2020). Available online at: https://www.reuters.com/article/health-coronavirus-china-jilin-idUSB9N2CG03K (accessed May 13, 2020).
29. Mcilroy T, Margo J. *Australia Adopts' Sentinel Testing' Regime to Beat Coronavirus*. (2020). Available online at: https://www.afr.com/politics/federal/australia-adopts-sentinel-testing-regime-to-beat-coronavirus-20200417-p54krd (accessed April 17, 2020).
30. Zhang X. Epidemiology of COVID-19. *N Engl J Med*. (2020) 382:1869–70. doi: 10.1056/NEJMc2005157
31. Gou F, Zhang X, Yao J, Yu D, Wei K, Zhang H, et al. Epidemiological characteristics of COVID-19 in Gansu province. *Chin J Epidemiol*. (2020). 41:E032. doi: 10.1142/9789811222078_0002
32. Fan J, Liu X, Shao G, Qi J, Li Y, Pan W, et al. The epidemiology of reverse transmission of COVID-19 in Gansu Province, China. *Travel Med Infect Dis*. (2020) 101741. doi: 10.1016/j.tmaid.2020.101741
33. Health Commission of Gansu Province. Three *New Confirmed Cases of COVID-19 Imported From Abroad China Have Been Reported in Gansu Province*. (2020). Available online at: http://wsjk.gansu.gov.cn/single/10910/84805.html (accessed March 14, 2020).

34. The Xinhua Agency. *Mandatory 14 Days Quarantine is Applied to Anyone Enter China for 24 Countries.* (2020). Available online at: http://www.xinhuanet.com/politics/2020-03/17/c_1125726374.htm (accessed March 19, 2020).
35. Shen S, Wei Y, Zhao Y, Jiang Y, Guan J, Chen F. Risk assessment of global COVID-19 imported cases into China. *Zhonghua Liu Xing Bing Xue Za Zhi*. (2020) 41:E070. doi: 10.3760/cma.j.cn112338-20200415-00577
36. The State Council of the People's Republic of China. *China to Enhance Epidemic Control in Key Areas, Improve Regular Prevention Measures.* (2020). Available online at: http://english.www.gov.cn/premier/news/202005/15/content_WS5ebe97f5c6d0b3f0e9497c1c.html (accessed May 15, 2020).
37. Birkhoff SD, Smeltzer SC. Perceptions of smartphone user-centered mobile health tracking apps across various chronic illness populations: an integrative review. *J Nurs Scholarsh.* (2017) 4:371–8. doi: 10.1111/jnu.12298
38. Health Commission of Gansu Province. One *New Confirmed Case of COVID-19 Imported From Hubei Province was Confirmed in Gansu Province.* (2020). Available online at: http://wsjk.gansu.gov.cn/single/10910/85032.html (accessed March 29, 2020).
39. Chen Y, Wang A, Yi B, Ding K, Wang H, Wang J, et al. The epidemiological characteristics of infection in close contacts of COVID-19 in Ningbo city. *Zhonghua Liu Xing Bing Xue Za Zhi.* (2020) 41:667–71. doi: 10.3760/cma.j.cn112338-20200304-00251
40. Varghese BM, Dent E, Chilver M, Cameron S, Stocks NP. Epidemiology of viral respiratory infections in Australian working-age adults (20-64 years): 2010-2013. *Epidemiol Infect.* (2018) 146:619–26. doi: 10.1017/S0950268818000286
41. Wang Y, Wang Y, Chen Y, Qin Q. Unique epidemiological and clinical features of the emerging 2019 novel coronavirus pneumonia (COVID-19) implicate special control measures. *Med Virol*. (2020) 92:568–76. doi: 10.1002/jmv.25748
42. Xiang F, Wang X, He X, Peng Z, Yang B, Zhang J, et al. (2020) Antibody detection and dynamic characteristics in patients with COVID-19. *Clin Infect Dis.* (2020) ciaa461. doi: 10.1093/cid/ciaa461
43. National Health Commission of People's Republic of China. *Pneumonia Infected With Novel Coronavirus is Included in the Management of Legal Infectious Diseases.* (2020). Available online at: http://www.nhc.gov.cn/jkj/s7915/202001/e4e2d5e6.f01147e0a8df3f6701d49f33.shtml (accessed January 31, 2020).
44. Tian H, Liu Y, Li Y, Wu C, Chen B, Kraemer M, et al. An investigation of transmission control measures during the first 50 days of the COVID-19 epidemic in China. *Science*. (2020) 368:638–42. doi: 10.1126/science.abb6105
45. The State Council of the People's Republic of China. *The Latest News on Epidemic Prevention and Control and Resumption of Work and Production on April 8*. (2020). Available online at: http://www.gov.cn/xinwen/2020-04/08/content_5500406.htm (accessed April 8, 2020).
46. Du N, Chen H, Zhang Q, Che L, Lou L, Li X, et al. A case series describing the epidemiology and clinical characteristics of COVID-19 infection in Jilin Province. *Virulence*. (2020) 11:482–5. doi: 10.1080/21505594.2020.1767357
47. National Health Commission of the People's Republic of China. *The Guideline for COVID-19 Prevention and Control (Sixth Edition).* (2020). Available online at: http://www.gov.cn/zhengce/zhengceku/2020-02/22/5482010/files/310fd7316a89431d977cc8f2dbd2b3e0.pdf (accessed February 21, 2020).
48. Health Commission of Gansu Province. *Change of Risk of New Coronavirus Pneumonia in Sub-Counties of Gansu province 2 Mar. 2020.* (2020). Available online at: http://wsjk.gansu.gov.cn/single/10910/84554.html (accessed March 2, 2020).
49. Health Commission of Jilin Province. *Notification on Adjusting the Emergency Response to COVID-19 From Top Level I to Level II to in Jilin Province.* (2020). Available online at: http://www.jl.gov.cn/zw/yw/jlyw/202002/t20200226_6861442.html (accessed February 2, 2020).
50. Eikenberry SE, Mancuso M, Iboi E, Phan T, Eikenberry K, Kuang Y, et al. To mask or not to mask: modeling the potential for face mask use by the general public to curtail the COVID-19 pandemic. *Infect Dis Model.* (2020) 5:293–308. doi: 10.1016/j.idm.2020.04.001
51. Esposito S, Principi N. To mask or not to mask children to overcome COVID-19. *Eur J Pediatr*. (2020) 179:1267–70. doi: 10.1007/s00431-020-03674-9

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Medicine

OPINION
published: 11 September 2020
doi: 10.3389/fmed.2020.00563

Science and the War on Truth and Coronavirus

*Geoffrey P. Dobson**

Heart, Trauma and Sepsis Research Laboratory, College of Medicine and Dentistry, James Cook University, Townsville, QLD, Australia

Keywords: science, coronavirus, truth, university, society, funding

I am a scientist and this is my brief on what the world needs to know about science and COVID-19. Science is a method of truth-telling about the physical world and ways to improve quality of life. It is the most powerful enterprise that has led to improved healthcare, a more sustainable environment, a safer world, and a better "knowing and understanding" about the world we live in. Science is fun and spectacular. And it has rarely let us down, until now.

Despite multiple warnings in 2015, the current global pandemic has revealed major deficits in our preparedness for a viral attack. Governments have let the public down by not supporting early warning programs and for not providing sufficient science funding to understand how different people respond differently to a viral attack, and vaccine development. The present pandemic has also revealed that science underpins a country's national security in ways never appreciated before. The resultant economic upheaval has thrown global supply chains, stock markets, the airline industry, oil markets, and the central bank into frenzied disarray (1). It is regrettable that it took a global pandemic, and the most powerful global economies to come crashing to their knees, with hundreds of thousands of lives lost, to bring science out of the shadows, and into the spotlight.

For decades, politicians have conveyed to the public how their country is leading science innovation and technology *but they fail to sufficiently support it*. In many areas, they are deaf to the calls from scientists, universities and research institutions to increase funding. Scientists themselves must also do a better job at explaining what they do and how science works. What most people don't understand is that science begins with a question and ends with a question (2). This is often confusing. If science is open-ended, how does it solve problems, like those from COVID-19? The short answer is you first have to understand what a virus is, where it has come from, how it enters the body, what it does when it gets there, and finally how to remove it. Finding answers to these questions raises more questions, and *it is this process of knowledge-building* and self-correction, that leads to improved understanding and development of new therapies, vaccines, and technological advances. Unlike bacteria that can thrive almost anywhere, a virus needs a living animal's cellular machinery for its replication and survival. Understanding how a virus has evolved the "tricks" to enter the body "undetected" is not fully understood. And if the COVID-19 virus enters our bodies, why do some people die a horrific death, others have flu-like symptoms, and 20 to 50% become asymptomatic carriers (3, 4)? And why do some children, a few weeks after contracting COVID-19, suffer a hyper-inflammatory attack and succumb to cardiovascular complications and toxic shock? (5).

Before science can answer questions on COVID-19, scientists need to better understand how the immune system works (6). The basic question on why some people have a very mild response, and others die from an explosive inflammatory attack is the 64-billion-dollar question. We have been working on *new drugs to bolster a patient's defense to a pathogen or injury, as part of the stress response, which we believe is controlled by the brain and resides at the intersection of the immune and inflammatory systems* (7, 8). While governments are spending billions of dollars developing a COVID-19 vaccine, we should not get complacent. Notwithstanding the global

OPEN ACCESS

Edited by:
Zisis Kozlakidis, International Agency for Research on Cancer (IARC), France

Reviewed by:
Romuald Polczyk, Jagiellonian University, Poland
Yunchang Shao, China National GeneBank (BGI), China

***Correspondence:**
Geoffrey P. Dobson
geoffrey.dobson@jcu.edu.au

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: *09 June 2020*
Accepted: *10 August 2020*
Published: *11 September 2020*

Citation:
Dobson GP (2020) Science and the War on Truth and Coronavirus. Front. Med. 7:563. doi: 10.3389/fmed.2020.00563

importance of developing a successful vaccine, it will not answer these, and related, fundamental scientific questions. Nor is a vaccine a substitute for the need to increase science funding, because history will repeat itself, another pandemic will occur, and the cycle will start over again. What we urgently need are new frontline drugs to blunt uncontrolled inflammation, and prevent pulmonary and cardiovascular dysfunction, coagulopathy and metabolic impairment (8–10).

I argue here that the current financial and health crisis is a symptom of a decade or more of budget cuts to basic scientific research, lack of job security among scientists, and declining interest in the next generation to pursue a career in science. Once a research grant is submitted to a funding body, the current success rate in the USA, Australia, UK and Europe is around 5 to 10%, so why facing these odds would a 12th grader or young university student want to become a scientist? The scientist and science, like teachers and nurses, continue to be undervalued by society. In the past decade, funding for the US National Institutes of Health (NIH) has steadily declined, losing around 20% of its funding capacity due to budget cuts, sequestration, and the impact of inflation. President Trump has proposed another 7% drop in NIH funding in 2021, and similar reductions to other science-based agencies (11). In Australia, government investment into research and development is at its lowest in 40 years (12). In Europe, a giant research programme, known as Horizon Europe, will be launched in 2021 across its 28 member states, and other countries, to fund Big Science involving large research groups (13), which leaves the individual scientist or small group of collaborators at a distinct disadvantage. It also remains to be seen, what percentage of those funds will be earmarked to support basic research, and early career scientists to set up their laboratories, who otherwise find it challenging to join large collaborative groups (13). Big Science is not the answer, and history has shown that most discoveries are made serendipitously by individual scientists thinking outside-the-box (2, 14, 15).

Notwithstanding the relentless hyperbole by government officials on funding, and their increasing attempts to pass the torch to industry, many scientists, universities and research institutions are in "survival mode" because of cut-backs. Universities are not businesses in a strict sense; they are involved in teaching and learning, research and technology, and job creation, which are designed to serve the needs of society. Industry, does, however, eventually benefit as the final receiver of potentially translatable products, but they are rarely the primary funders. *Hopefully, the current pandemic will drive home to politicians and lawmakers the societal role of a university, and that the current funding schemes are not fit for purpose.*

Another critical aspect of science is that a "truth" or "fact" in science is an evidence-based statement, not just a "subjective" feeling or an impression (2). When President Donald Trump told the world that he thinks the antimalarial drug hydroxychloroquine is safe and that he would take it, is not an evidence-based statement. An evidence-based statement needs to be tested using the tools of science and medicine, which involves some kind of effect, measurement, human trials and peer review. That CO_2 in our atmosphere is rising has also sparked a lot of political and public confusion with mixed messages. The *preponderance of evidence* from the vast majority of scientists *specializing in this research* conclude that the rise in CO_2 is associated with global warming and is accelerated from the burning of fossil fuels, deforestation and human activity (16). Of course, there are critics, however, *the preponderance of evidence* suggests that time is running out, and the warnings that are eerily similar to those leading up to the present pandemic. Unexpectedly, the current global shutdown in early 2020 has also provided us with a global experiment in reducing greenhouse gas emissions. Some countries like China decreased emissions by up to 25%, and the people in India can now see the snow-capped peaks of the Himalayas for the first time in decades (17). It is important to remember that the statement "The rise in CO_2 is associated with global warming and human activity" is not an absolute statement–it is based on the preponderance of available evidence. Science does not deal with absolutes or first causes, which is its power not its weakness. A provisional-based knowledge allows science to self-correct with improved "truths" and deliverables. This provisional nature of science is often used to attack the process in the media, which sends mixed messages to the public and politicians.

Since February 2020 I have never heard the word "science" mentioned so many times in my 30 years as a scientist, or have I witnessed its credibility being blindly attacked for political gain. We live in a dangerous world and we are outnumbered; 20 million viruses can fit on the head of pin. We need to embrace these new realities, listen to the experts, and not be swayed by the uninformed or naysayers (18–20). Now is a pivotal time in history. I hope the current COVID pandemic has exposed major gaps in Government funding of basic science, and that they stop throwing out pocket-change to scientists thinking that the problem will go away. If we do not learn from our mistakes, I fear 100 years from now historians will write: "the people of the early twenty-first century remained imprisoned by the past and failed to embrace the tools to break free." Breaking free requires a new global stewardship, with new partnership programs in education, increased funding of basic science and technology, and a renewed optimism that anything is possible.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

ACKNOWLEDGMENTS

The author would like to thank Professor Jakob Vinten Johansen and the JCU research team Drs. Hayley Letson, Jodie Morris, and Erik Biros for many discussions on the topic. GD would particularly like to thank the US Department of Defense (USSOCOM) for their continued support of our work and the College of Medicine and Dentistry at James Cook University.

REFERENCES

1. Sharif A, Aloui C, Yarovaya L. COVID-19 pandemic, oil prices, stock market, geopolitical risk and policy uncertainty nexus in the US economy: fresh evidence from the wavelet-based approach. *Int Rev Financial Analysis*. (2020) 70:101496. doi: 10.1016/j.irfa.2020.101496
2. Dobson GP. *A Chaos of Delight: Science, Religion and Myth and the Shaping of Western Thought*. Oakville, CT: David Brown and Co (2005).
3. Abbasi J. Anthony Fauci,MD, on COVID-19 vaccines, schools, and larry kramer. *JAMA*. (2020) 1:E1–3. doi: 10.1001/jama.2020.9222
4. Shi S, Qin M, Shen B, Cai Y, Liu T, Yang F, et al. Association of cardiac injury with mortality in hospitalized patients with COVID-19 in Wuhan, China. *JAMA Cardiol*. (2020) 5:802–10. doi: 10.1001/jamacardio.2020.0950
5. Viner RM, Whittaker E. Kawasaki-like disease: emerging complication during the COVID-19 pandemic. *Lancet*. (2020) 395:1741–3. doi: 10.1016/S0140-6736(20)31129-6
6. Callaway E, Ledford H, Mallapaty S. Six months of coronovirus: the mysteries scientists are still racing to solve. *Nature*. (2020) 583:178–9. doi: 10.1038/d41586-020-01989-z
7. Dobson GP. Trauma of major surgery: a global problem that is not going away. *Int J Surg*. (2020) 47–54. doi: 10.1016/j.ijsu.2020.07.017
8. Dobson GP, Morris JL, Davenport LM, Letson HL. Traumatic-induced coagulopathy as a systems failure: a new window into hemostasis. *Semin Thromb Hemost*. (2020) 46:199–214. doi: 10.1055/s-0039-1701018
9. Liu Q, Zhou YH, Yang ZQ. The cytokine storm of severe influenza and development of immunomodulatory therapy. *Cell Mol Immunol*. (2016) 13:3–10. doi: 10.1038/cmi.2015.74
10. Feldmann M, Maini RN, Woody JN, Holgate ST, Winter G, Rowland M, et al. Trials of anti-tumour necrosis factor therapy for COVID-19 are urgently needed. *Lancet*. (2020) 395:1407–9. doi: 10.1016/S0140-6736(20)30858-8
11. Kaiser J. National Institutes of Health would see 7% cut in 2021 under white house plan. *Science*. (2020) 367:15. doi: 10.1126/science.abb2481
12. Nogrady B. Australia cuts research funding for universities. *Nature*. (2018) 25–6. doi: 10.1038/d41586-018-07840-w
13. Abbott A, Schiermeier Q. How European scientists will spend €100 billion. *Nature*. (2019) 569:472–5. doi: 10.1038/d41586-019-01566-z
14. Valentine AJ. Comment on 'Big science, little science'. *EMBO Rep*. (2010) 11:152. doi: 10.1038/embor.2010.16
15. Foletti A, Fais S. Unexpected discoveries should be reconsidered in science—a look to the past? *Int J Mol Sci*. (2019) 20:3973. doi: 10.3390/ijms20163973
16. Coutts C, Hahn M. Green infrastructure, ecosystem services, and human health. *Int J Environ Res Public Health*. (2015) 12:9768–98. doi: 10.3390/ijerph120809768
17. Gautam S. The influence of COVID-19 on air quality in India: a boon or Inutile Sneha Gautam. *Bull Environ Contam Toxicol*. (2020) 104:724–6. doi: 10.1007/s00128-020-02877-y
18. Calisher C, Carroll D, Colwell R, Corley RB, Daszak P, Drosten C, et al. Statement in support of the scientists, public health professionals, and medical professionals of China combatting COVID-19. *Lancet*. (2020) 395:e42–3. doi: 10.1016/S0140-6736(20)30418-9
19. Ball P, Maxmen A. The epic battle against coronavirus misinformation and conspiracy theories. *Nature*. (2020) 581:371–4. doi: 10.1038/d41586-020-01452-z
20. Larson HJ. A call to arms: helping family, friends and communities navigate the COVID-19 infodemic. *Nat Rev Immunol*. (2020) 20:449–50. doi: 10.1038/s41577-020-0380-8

Conflict of Interest: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pediatrics

BRIEF RESEARCH REPORT
published: 11 September 2020
doi: 10.3389/fped.2020.00547

Seroprevalence of SARS-CoV-2 Among Pediatric Healthcare Workers in Spain

Ana Dacosta-Urbieta[1,2], Irene Rivero-Calle[1,2], Jacobo Pardo-Seco[1,2,3], Lorenzo Redondo-Collazo[1,2], Antonio Salas[1,3], Jose Gómez-Rial[1,4] and Federico Martinón-Torres[1,2†]*

[1] Grupo de Investigación en Genética, Vacunas, Infecciones y Pediatría (GENVIP), Instituto de Investigación Sanitaria de Santiago (IDIS) and Hospital Clínico Universitario and Universidade de Santiago de Compostela (Servicio Gallego de Salud), Galicia, Spain, [2] Translational Pediatrics and Infectious Diseases, Department of Pediatrics, Hospital Clínico Universitario de Santiago de Compostela, Galicia, Spain, [3] Unidade de Xenética, Instituto de Ciencias Forenses (INCIFOR), Facultade de Medicina, Universidade de Santiago de Compostela, and GenPoB Research Group, Instituto de Investigaciones Sanitarias (IDIS), Hospital Clínico Universitario de Santiago (Servicio Gallego de Salud), Galicia, Spain, [4] Laboratorio de Inmunología, Servicio de Análisis Clínicos, Hospital Clínico Universitario Santiago de Compostela (Servicio Gallego de Salud), Galicia, Spain

OPEN ACCESS

Edited by:
Valeriane Leroy, Institut National de la Santé et de la Recherche Médicale (INSERM), France

Reviewed by:
Tatjana Vilibic-Cavlek, Croatian Institute of Public Health, Croatia
Guillermo Soza, University of La Frontera, Chile

***Correspondence:**
Federico Martinón-Torres
federico.martinon.torres@sergas.es

†ORCID:
Federico Martinón-Torres
orcid.org/0000-0002-9023-581X

Specialty section:
This article was submitted to Pediatric Infectious Diseases, a section of the journal Frontiers in Pediatrics

Received: *13 May 2020*
Accepted: *29 July 2020*
Published: *11 September 2020*

Citation:
Dacosta-Urbieta A, Rivero-Calle I, Pardo-Seco J, Redondo-Collazo L, Salas A, Gómez-Rial J and Martinón-Torres F (2020) Seroprevalence of SARS-CoV-2 Among Pediatric Healthcare Workers in Spain. Front. Pediatr. 8:547. doi: 10.3389/fped.2020.00547

Spain is one of the countries most severely affected by the SARS-CoV-2 pandemic, with almost 190,000 cases as of April 18, 2020. As healthcare workers (HCW) are one of the groups hardest hit by the infection, it is important to know the seroprevalence of antibodies against SARS-CoV-2 in pediatric departments. We performed 175 immunoglobulin (Ig)M and IgG immunochromatographic rapid tests in the personnel working at the Pediatric Department of the Hospital Clínico Universitario of Santiago de Compostela (Spain), including pediatricians, residents, nurses, and other staff, on days 31–33 since the lockdown started. Seven out of the 175 tests were positive, including four for IgM and three for IgG, leading to a seroprevalence of 4.0% (95% CI: 1.1–6.9%). Only one of them had symptoms at the time of testing (sore throat). All seropositive cases yielded negative RT-PCR of the upper and lower respiratory tract. This is the first SARS-CoV-2 serological survey among HCWs reported in Spain. Notwithstanding the test limitations, our results reveal that personal protection policy and lockdown measures have been effective to limit population exposure. The low seroprevalence rate poses a significant challenge for the next strategic steps of pandemic control.

Keywords: seroprevalance, COVID-19, healthcare worker (HCW), SARS-CoV-2, rapid test for COVID-19

INTRODUCTION

Spain is one of the countries with the highest number of cases of SARS-CoV-2 reported worldwide, with almost 190,000 cases confirmed as of April 18, 2020 (1). The first case in Spain was recorded on January 31, and the Spanish government started the lockdown on March 14, when the country had 7,753 active cases. In Galicia (northwest Spain), the first case was recorded on the March 4, and at the moment of the lockdown, there were 195 confirmed cases. In the last 2 weeks, the cumulative incidence of SARS-CoV-2 is 149.61 cases/100,000 inhabitants in Spain, and 103.5 cases/100,000 inhabitants (1) in Galicia. To date, we have identified 19 pediatric cases in our hospital, and only one of them was admitted to hospital for reasons unrelated to the infection. We aimed to assess the seroprevalence rate of SARS-CoV-2 among healthcare workers (HCWs) of the Pediatric Department of the Hospital Clínico Universitario de Santiago de Compostela, the reference hospital in Galicia.

TABLE 1 | Characteristics of the seropositive subjects.

	Subject 1	Subject 2	Subject 3	Subject 4	Subject 5	Subject 6	Subject 7
Place of work	PW	PICU	PC	NICU	NICU	PW	PW
Staff category	NA	Nurse	Pediatrician	Nurse	RP	RP	NA
Age (years)	55–64	35–44	55–64	35–44	25–34	25–34	35–44
Exposure	No	No	Yes	No	Yes	No	No
House contacts	Yes	Yes	Yes	Yes	Yes	No	Yes
Pets at home	No	No	–	No	No	No	No
Comorbidities	No	No	–	Yes	Yes	No	No
Symptoms	No	No	No	No	No	No	Yes
Test result	IgM+/RT-PCR–	IgM+/RT-PCR–	IgM+/RT-PCR–	IgM+/RT-PCR–	IgG+/RT-PCR–	IgG+/RT-PCR–	IgG+/RT-PCR–
Treatment received	No	No	No	No	No	No	No

PW, pediatric ward; PC, primary care; RP, pediatrics resident; NA, nurse aide; PCR–, RT-PCR negative; Exposure, exposure to SARS-CoV-2-positive patients; Symptoms, symptoms suggestive of SARS-CoV-2; Ig, immunoglobulin; PICU, pediatric intensive care unit; NICU, neonatal intensive care unit.

METHODS

We performed a sero-epidemiological survey on days +31, +32, and +33 after lockdown started, including all HCWs of the Pediatric Department of the Hospital Clínico Universitario de Santiago de Compostela, namely, pediatricians, resident doctors, nurses, and administrative staff. Emergency Department and the Pediatric Intensive Care Unit were considered the most exposed areas.

We tested immunoglobulin (Ig)M and IgG against SARS-CoV-2 by an immunochromatographic rapid method (Virusee® by Genobio Pharmaceutical, Shanghai, China) using 10-μl finger-prick/capillary blood. Sensitivity and specificity rates specified by the manufacturer were 96.0% and 96.8% for IgG and 94.6% and 96.8% for IgM detection, respectively. The rapid test used provides the result in 10 min. Epidemiological, clinical, and laboratory data were collected in all cases.

RESULTS

The test was performed on 175 HCWs. Most of them were pediatric consultants (32.6%; $n = 57$), followed by nurses and nurses' aides (47.4%; $n = 83$), pediatrics residents (13.1%; $n = 23$), and others (6.9; $n = 12$). In addition, 18.3% ($n = 32$) of the workers were ≥55 years old, and 12.6% ($n = 22$) had preexisting comorbid conditions such as asthma (4.0%; $n = 7$), high blood pressure (1.7%; $n = 3$), or obesity (1.7%; $n = 3$). Moreover, 22.5% of the workers had a known exposure to SARS-CoV-2-positive patients. When asked if they recalled any symptoms suggestive of SARS-CoV-2 in the last 2 months, 53% of the workers declared none.

Seven workers yielded positive test results, three of them were IgG positive and four of them were IgM positive, representing a total seroprevalence of 4.0% of the cohort (95% CI: 1.1–6.9%) (**Table 1**). The subjects who tested positive for IgM worked in the most exposed areas were: two medical residents and one consultant, two nurses, and two nurse aides. None of the workers who tested positive for IgM presented symptoms at the time of the test. Of the subjects who tested positive for IgG, only one of them recalled symptoms suggestive of coronavirus infection (cough and sore throat), and these persisted at the time of testing. In the positive cases, RT-PCR was performed on the lower (oropharyngeal swab) and upper respiratory tracts (nasal swab), all with negative results.

DISCUSSION

To date, there are few data regarding serological responses to SARS-CoV-2 in infected patients (2) and virtually no data on serological responses to SARS-CoV-2 in asymptomatic exposure. Rapid tests using serology have the advantage of delivering quick results and allowing the testing of asymptomatic people reliably (3); the technique is easier and quicker than RT-PCR in respiratory samples. True sensitivity and specificity can vary depending on the commercial test, and this fact warrants the need for further studies. Despite the limitations of the rapid test applied, the limited number of subjects analyzed, and the lack of confirmatory alternative serological assays, the present study indicates a low exposure to SARS-CoV-2 among pediatric HCWs in Spain. There is only one other serologic study published in Spain (4) where the total number of participants with evidence of past or current infection (by PCR and/or serology) was 11.2% (65/578); however, these results come from a large referral hospital in Barcelona, Spain, one of the regions with the highest burden of disease of coronavirus disease 2019 (COVID-19) in Spain.

Although our results must be interpreted with caution since it is possible that some of the HCWs with IgG positivity could have acquired the infection outside the hospital, the low seroprevalence against SARS-CoV-2 among pediatric HCWs points to the success of personal protection and lockdown policies together with a low nosocomial risk of infection, while highlighting the challenge for the next stages of SARS-CoV-2 pandemic management.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comité de Ética de la investigación con medicamentos de Galicia (CEIm-G). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

This study received support from the Instituto de Salud Carlos III: project GePEM (Instituto de Salud Carlos III(ISCIII)/PI16/01478/Cofinanciado FEDER), DIAVIR (Instituto de Salud Carlos III(ISCIII)/DTS19/00049/Cofinanciado FEDER; Proyecto de Desarrollo Tecnológico en Salud) and Resvi-Omics (Instituto de Salud Carlos III(ISCIII)/PI19/01039/Cofinanciado FEDER) and project BI-BACVIR (PRIS-3; Agencia de Conocimiento en Salud (ACIS)-Servicio Gallego de Salud (SERGAS)-Xunta de Galicia; Spain) given to AS and project ReSVinext (Instituto de Salud Carlos III(ISCIII)/PI16/01569/Cofinanciado FEDER), and Enterogen (Instituto de Salud Carlos III(ISCIII)/ PI19/01090/Cofinanciado FEDER) given to FM-T.

REFERENCES

1. Ministerio de Sanidad, Gobierno de España. Actualización n° 78. *Enfermedad Por el Coronavirus (COVID-19)*. (2020). Available online at: https://www.mscbs.gob.es/profesionales/saludPublica/ccayes/alertasActual/nCov-China/documentos/Actualizacion78_COVID-19.pdf (accessed April 17, 2020).
2. Zhao J, Yuan Q, Wang H, Liu W, Liao X, Su Y, et al. Antibody responses to SARS-CoV-2 in patients of novel coronavirus disease 2019. *Clin Infect Dis.* (2020) 28:ciaa344. doi: 10.1093/cid/ciaa344
3. Li Z, Yi Y, Luo X, Xiong N, Liu Y, Li S, et al. Development and clinical application of a rapid IgM-IgG combined antibody test for SARS-CoV-2 infection diagnosis. *J Med Virol.* (2020). doi: 10.1002/jmv.25727. [Epub ahead of print].
4. García-Basteiro AL, Moncunill G, Tortajada M, Vidal M, Guinovart C, Jiménez A, et al. Seroprevalence of antibodies against SARS-CoV-2 among health care workers in a large Spanish reference hospital. *Nat Commun.* (2020). 11:3500. doi: 10.1101/2020.04.27.20082289

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Public Health

REVIEW
published: 15 September 2020
doi: 10.3389/fpubh.2020.567184

Seasonality of Respiratory Viral Infections: Will COVID-19 Follow Suit?

Amani Audi[1,2], *Malak Allbrahim*[1,2†], *Malak Kaddoura*[1,2], *Ghina Hijazi*[1,2], *Hadi M. Yassine*[3*] *and Hassan Zaraket*[1,2*]

[1] Department of Experimental Pathology, Immunology & Microbiology, Faculty of Medicine, American University of Beirut, Beirut, Lebanon, [2] Faculty of Medicine, Center for Infectious Disease Research, American University of Beirut, Beirut, Lebanon, [3] Biomedical Research Center and College of Health Sciences-QU Health, Qatar University, Doha, Qatar

Respiratory viruses, including coronaviruses, are known to have a high incidence of infection during winter, especially in temperate regions. Dry and cold conditions during winter are the major drivers for increased respiratory tract infections as they increase virus stability and transmission and weaken the host immune system. The novel severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) emerged in China in December 2020 and swiftly spread across the globe causing substantial health and economic burdens. Several countries are battling with the second wave of the virus after a devastating first wave of spread, while some are still in the midst of their first wave. It remains unclear whether SARS-CoV-2 will eventually become seasonal or will continue to circulate year-round. In an attempt to address this question, we review the current knowledge regarding the seasonality of respiratory viruses including coronaviruses and the viral and host factors that govern their seasonal pattern. Moreover, we discuss the properties of SARS-CoV-2 and the potential impact of meteorological factors on its spread.

Keywords: coronaviruses, COVID-19, severe acute respiratory syndrome coronavirus-2, respiratory viruses, seasonality, temperature, humidity

OPEN ACCESS

Edited by:
Zhongheng Zhang,
Sir Run Run Shaw Hospital, China

Reviewed by:
Makoto Takeda,
National Institute of Infectious Diseases (NIID), Japan
Catherine Ropert,
Federal University of Minas Gerais, Brazil

***Correspondence:**
Hassan Zaraket
hz34@aub.edu.lb
Hadi M. Yassine
hyassine@qu.edu.qa

†ORCID:
Malak Allbrahim
orcid.org/0000-0002-8801-5228

Specialty section:
This article was submitted to Infectious Diseases, Surveillance, Prevention and Treatment, a section of the journal Frontiers in Public Health

Received: *29 May 2020*
Accepted: *27 August 2020*
Published: *15 September 2020*

Citation:
Audi A, Allbrahim M, Kaddoura M, Hijazi G, Yassine HM and Zaraket H (2020) Seasonality of Respiratory Viral Infections: Will COVID-19 Follow Suit? Front. Public Health 8:567184. doi: 10.3389/fpubh.2020.567184

INTRODUCTION

The severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) is the third zoonotic and highly pathogenic coronavirus (CoV) to emerge in the twenty-first century (1). The earliest cases of SARS-CoV-2 infections were reported in December 2019 in Wuhan, Hubei Province, China, the epicenter of the outbreak (1). Since then, the virus has been rapidly spreading across the globe (2).

CoVs are a large group of positive-stranded RNA viruses that commonly infect birds and mammals, causing a wide range of pathological conditions (3). These viruses undergo frequent mutations and recombinations, yielding new variants that can cross the species barrier (3). Since 1960, seven coronaviruses (CoVs) have been identified to cause infections among humans (4). Human coronaviruses (HCoV) 229E, OC43, HKU1, and NL63 are common in the human population and are responsible for about 15–30% of the annual respiratory tract infections (5). They are commonly associated with mild and self-limiting symptoms. Still, severe illnesses, accompanied by lower respiratory tract infection, might also occur, especially in elderly, neonates, and patients with underlying health conditions and risk factors (5).

In the current millennium, three highly pathogenic CoVs, SARS-CoV-1 (6), the Middle East respiratory syndrome CoV (MERS-CoV) (7), and the recently emerged SARS-CoV-2 (1),

have crossed the species barrier and resulted in human infections. SARS-CoV-1 was first detected in Guangdong Province, China, in November 2002 and then rapidly spread to Hong Kong and 29 other countries, resulting in more than 8000 confirmed cases, including 774 deaths (6, 8). By July 2003, the virus died out throughout the world. MERS-CoV was first detected in Saudi Arabia in 2012, with the camels being the source for human infections (9). The virus caused a total of 2,519 laboratory-confirmed cases, including 866 associated deaths as of the end of January 2020 (7). The majority of cases were detected in the Kingdom of Saudi Arabia (KSA), in addition to one major outbreak in South Korea (10).

SARS-CoV-2 is a highly contagious virus that is associated with severe pneumonia cases (11). On January 30, 2020, the World Health Organization (WHO) announced COVID-19 (coronavirus infectious disease) as Public Health Emergency of International Concern after it affected 7,818 people with 170 deaths in 19 countries, including China (12). Since late February, the number of reported COVID-19 cases along with the number of affected countries had sharply increased within a short period, which led the WHO to declare the global COVID-19 outbreak a pandemic (13). Since then, the number of globally confirmed COVID-19 cases has been increasing exponentially, resulting in nearly more than 21 million confirmed cases and 761,000 fatalities as of August 16, 2020 (14).

Different approaches and interventions have been adopted to contain and control the disease spread including travel restrictions (global), partial or complete lockdowns (e.g., China and Singapore) (15, 16) and/or massive testing and isolation of confirmed cases and their contacts (South Korea) (17). The reluctance and delayed implementation of multilayered public health measures in some countries (e.g., Italy, Iran, the UK, Brazil, and the US) resulted in dire outcomes. Despite all the efforts and measures to contain the virus, it is still spreading globally, traversing all climate and environmental settings (2).

Nearly every acute viral disease has a particular seasonal window of occurrence, which differs according to the geographic location and environmental conditions (18). The incidence of respiratory viral infections is highly affected by seasonal changes, especially in temperate climates (19). Extensive research has been done to better understand the seasonality of respiratory viruses. Yet, our knowledge about this phenomenon remains limited. Here we attempt to address the possible impact of weather on SARS-CoV-2 spread, taking into consideration the current knowledge regarding its stability and transmission patterns, and the behavior of other respiratory viruses.

SEASONALITY OF CORONAVIRUSES AND OTHER RESPIRATORY VIRUSES

Most viral respiratory infections tend to follow seasonal patterns with high incidence during winter in temperate regions and during the rainy season in tropical regions (20). Influenza virus and respiratory syncytial virus (RSV) have a single annual seasonal peak during winter in the Northern and Southern temperate regions (21). These viruses peak from December to March in the Northern hemisphere, and between June and August in the Southern hemisphere (21). Parainfluenza viruses have a seasonal peak from April to June in the Northern temperate sites and during September in the Southern temperate areas (21). In most of the tropical regions, these viruses occur year-round with increased incidence in rainy seasons (21, 22). Rhinoviruses and adenoviruses, two non-enveloped respiratory viruses, are known to circulate throughout the year in all climatic regions with occasional peaks in autumn and winter for rhinoviruses and in winter and early spring for adenoviruses (23, 24).

Epidemiologic studies of common cold HCoVs suggest that they exhibit a seasonal pattern. In a temperate climate, HCoV infections are primarily detected in winter and spring, with low-level circulation throughout the year (3). The early known types, HCoV-OC43 and HCoV-229E, predominantly circulate during the winter season in temperate climate countries (25, 26). An eight-year study of HCoV-OC43 and HCoV-229E among young adults in the US reported an equal number of infections with these two types during the winter (December through February) and spring season (March through May) (27). In Belgium, HCoV-OC43 and HCoV-229E were only detected in winter and early spring (28). Several other studies from the United States, Belgium, France, Canada, Japan, Jordan, Italy, and Germany consistently reported winter circulation of the other two HCoVs: NL63 and HKU1 (28–36).

On the other hand, tropical/subtropical regions display year-round circulation of HCoVs but with increased activity during certain months. A study conducted in China during 2008–2009 reported that HCoV-NL63 and HCoV-HKU1 infections, in hospitalized children with acute respiratory infections, showed increased activity during summer, fall, and winter (37). In another 7-year epidemiologic study between 2009 and 2016 in China, HCoVs circulated year-round but with the highest incidence during the spring and autumn (38). A study by Chiu et al., in Hong Kong, showed that HCoV-NL63 infections were notable during the spring and summer months of 2002, whereas HCoV-OC43 infections mainly occurred during the fall and winter of 2001 (39). Additionally, a study from Thailand confirmed the previous findings and reported the peak of HCoV-OC43 activity in winter, whereas HCoV-NL63 frequently occurred in autumn (40). In Australia, HCoV-NL63 peaks in mid-winter but was also detected between late-autumn and early-spring (41). Studies from some African countries (South Africa and Ghana) also reported a year-round circulation of HCoVs (42, 43).

Despite its rapid spread to about 30 countries, the SARS-CoV-1 was quickly contained. Thus, it was not possible to assess its seasonality. In the case of MERS-CoV, seven years have passed since its emergence and is still causing intermittent and sporadic infections without obvious seasonality (10). In fact, MERS-CoV has demonstrated low ability to transmit between humans, and most of the outbreaks have occurred mainly in healthcare settings. In camels, the virus seems to peak between late-winter and early-summer (44). This coincides with a spike in zoonotic transmission between April and July (45). A 5-year epidemiologic study, conducted between 2012 and 2017,

demonstrated that MERS-CoV has the highest global seasonal occurrence during June with some observed seasonal variations (46). A case-cross-over analysis of the associations between primary human MERS cases and weather conditions found that the primary MERS infections are more likely to occur in cold and dry conditions (47).

In summary, most respiratory viruses follow a seasonal pattern. However, some factors might increase the incidence of these infections, even in seasons with low circulation. For instance, an increased incidence of respiratory infections occurs among pilgrims during the Hajj Season (48). The mass crowding in a limited space, in addition to the close contact between pilgrims, increases the risk of viral importation and transmission, particularly the respiratory ones (49) Rhinovirus, influenza virus, and the common cold HCoVs (mainly HCoV-229E) are usually the most commonly detected viruses during the Hajj (48).

DRIVERS OF SEASONALITY OF RESPIRATORY VIRUSES

Seasonality of viral respiratory infections can be primarily attributed to two main factors: the environmental and weather effects on the virus and the host, as well as the host's behavior and physiology (20). Studies on respiratory viruses, including influenza viruses, suggest that cold weather and low relative humidity are highly associated with the onset of respiratory infections in the temperate regions (50, 51). This was mainly attributed to the effect of temperature and humidity on the stability and transmissibility of the viral particles, in addition to the effect on the host airway immune response (19).

Effect of Meteorological Factors on the Stability and Transmission of Respiratory Viruses

A study by Price et al. demonstrated that unlike the non-enveloped viruses that circulate throughout the year, enveloped viruses, including influenza and RSV, tend to be more seasonal, with a clear preference for colder temperatures (20). Harper et al. found that the optimal airborne influenza survival is at low temperatures and the survival decreases as the temperature increases (52). Low temperatures seem to enhance the lipid ordering of the viral envelope and improve influenza virus stability (53). This enhances the virus's ability to stay protected outside the body for a longer period of time (54). Further, a systematic review examined the factors that affect influenza survival on different metrics revealed that longer virus survival is favored at lower temperatures (55).

Besides their effect on stability, low temperature and relative humidity are also shown to enhance aerosol transmission of respiratory viruses (52). It was proposed that influenza virus transmission occurs mostly by aerosols in temperate regions and by contact in tropical sites (56). Using the guinea pig model, Lowen et al. showed that influenza virus aerosol transmission is suppressed by high humidity and warm temperature, but enhanced under cold and dry conditions (57). Low relative humidity induces evaporation of water from the exhaled bio-aerosols, leading to the formation of droplet nuclei (1–5 μm in size) (58). The extent of infectious viral particles survival in dried aerosols is not known; however, it is speculated that these nuclei can stay suspended in the air for prolonged periods (58). The opposite happens at high relative humidity, whereby the respiratory droplets increase in size by taking on water from the surrounding and quickly settle out of the air, thus, decrease aerosol transmission of the virus (58).

On the other hand, the transmission of influenza viruses by contact was shown to be efficient even at high humidity (54). High humidity enhances the indirect virus transmission by increasing the virus particle's stability, inside droplets, on surfaces (54). A study by Yang et al. showed that humidity promotes the survival of influenza A virus by controlling the extent of evaporation in these virus-containing droplets, which affect the solute concentrations and thus, viral stability (59). This partially explains the year-round occurrence of viral respiratory infections in tropical regions, particularly during rainy seasons when humidity is high.

In addition, it is well-known that solar UV radiation (UV) of all wavelengths effectively inactivate RNA and DNA viruses to varying extents (60, 61). Three types of UV radiations, UVA, UVB, and UVC, exist in nature, with UVC, having the shortest wavelength range, being the most effective against viruses (62). However, only UVA and UVB radiations are found at the ground-level sunlight, and these are known to have lower efficiency against viruses (60). The low incidence of respiratory infections during summer in temperate regions can also be attributed to the solar inactivation of viruses on the outdoor surfaces contaminated with respiratory secretions, thus decreasing the possibility of fomite transmission.

Effect of Meteorological Factors on Host's Susceptibility to Infection

Meteorological or environmental conditions were also shown to have a direct effect on the host's susceptibility to infections (63). The role of cold weather in weakening the immune response is controversial (63, 64). However, many studies indicated that cold and dry environments have an immunosuppressive effect on the host, and thereby increase the risk of acquiring infections (65–67). Increased exposure to cold air was shown to induce a temperature-related reduction in lung function in patients with chronic inflammatory airway diseases, such as chronic obstructive pulmonary disease (COPD) and asthma (68). Seasonal changes in temperature were also shown to affect the local immune response in the nose (66). It was shown that the antiviral defense response against rhinovirus infection in cultured mouse airway cells is reduced at low temperature (69). The cooling of the nasal airway by the inhaled cold air induces a decrease in the temperature of the respiratory epithelium, and compromise both the mucociliary clearance (MCC) in the nose and the local immune response in the upper airway (66).

The nasal respiratory epithelium is made up of ciliated cells covered with an airway surface layer comprised of a mucus layer that catches inhaled particles and low viscosity pericilliary

layer that moisturizes the surfaces and enable ciliary beating (70). MCC is a key mechanism required for getting rid of particles, including infectious agents, stuck on the surface of the respiratory epithelium (70). Production of thin mucosal layer and beating of cilia at a specific frequency are considered key factors for efficient MCC (66). MCC was shown to be affected by temperature and relative humidity. A recent study demonstrated that MCC and epithelial cellular repair in influenza virus-infected cells is reduced at a low relative humidity (67). Using a climate chamber for cell culture, a study showed that a temperature of 25°C and RH of 40% induced more production of mucin compared to 37°C and 80% RH (71). In addition, it was shown that tracheal and nasal mucociliary beat frequency decreases as the temperature falls below 20°C and totally ceases at 5°C (72). These studies indicate that low temperature and low humidity in the nasal airway compromise the MCC by increasing mucin secretion and reducing mucociliary beat frequency (67, 71, 73). Moreover, a study done on guinea pigs revealed that breathing dry air can disrupt cilia, damage epithelial cells, and induce local inflammation of the trachea (74). More importantly, the phagocytic activity of macrophages, a key non-specific immune response mechanism against viruses, was found to be reduced both *in vivo* and *in vitro* at low temperatures (75).

It has also been postulated that shortened exposure to sunlight during the winter affects vitamin D levels, a key modulator for both innate and adaptive immune responses, which increases the susceptibility to respiratory infections during winter (76, 77). A systematic review assessing the relation between vitamin D and respiratory tract infections found that vitamin D supplementation reduces the incidence of respiratory tract infections (78). The high incidence of influenza was also correlated with the seasonal decrease in vitamin D levels (79). A recent observational study of 212 patients from three South Asian hospitals, found a positive association between vitamin D levels and clinical outcomes of COVID-19 patients (80).

MODES OF SARS-CoV-2 TRANSMISSION

The respiratory transmission mode of SARS-CoV-2 is not fully understood. However, the virus is assumed to have a transmission pattern similar to that of the influenza virus (81). These modes include transmission through direct or indirect contact with infected individuals. Transmission of the virus can occur via fomites or direct contact with an infected person or through respiratory droplets released during sneezing, coughing, or talking (82). Studies showed that SARS-CoV-2 can stay viable on surfaces for hours or even days especially in healthcare facilities where the concentration of the virus released by the patients is relatively high (82–84). The survival of the virus on these surfaces depends on relative humidity and temperature and on the nature of the contaminated surfaces (85, 86).

Although airborne virus transmission has not yet been confirmed in humans, studies suggest that the occurrence of aerosol transmission cannot be excluded, especially in closed venues (82, 87). Airborne transmission occurs when the aerosols (droplet nuclei $<5\,\mu m$) containing infectious viral particles spread in air over a long distance and remain suspended for a long time (82). These aerosols are produced from evaporation of large respiratory droplets or released from the infected individuals by coughing, sneezing, talking, or exhaling. The aerosols can be breathed by individuals and cause infection if enough infectious dose of the virus is present or upon extended exposure (82). A study by Van Dormalan et al. found that SARS-CoV-2 virus particles remained infectious for 3 h in experimentally generated virus-containing aerosols that mimic the human-generated ones (83). Several studies reported detecting SARS-CoV-2 RNA in the air samples collected from different areas inside the hospitals such as patients' toilet areas, medical staff areas, and public areas prone to crowding (83, 88, 89). Recently, it was shown that infectious SARS-CoV-2 can be detected in air samples collected 2–4.8 m away from hospitalized COVID-19 patients, supporting the possibility of airborne transmission at least in confined environments (90).

The possibility of transmission via the fecal-oral or fecal-respiratory route has been also considered for COVID-19. SARS-CoV-2 RNA and viable virus were also found in urine and feces of infected patients (91–95). However, no evidence on virus transmission through feces or urine exists (91–95). Some studies also reported the detection of SARS-CoV-2 RNA but not infectious virus in blood samples of COVID-19 patients and breast milk of infected mothers (91, 96, 97). The absence of viable virus in blood and breast milk excludes the possibility of virus transmission through these routes (91, 96, 97).

Controlling the transmission of respiratory viruses is very challenging on its own, but is even more complicated in the case of SARS-CoV-2 due to the well-demonstrated role of asymptomatic or pre-symptomatic carriers (98–101). A meta-analysis of nine studies from six countries (including 21,035 close contacts of 843 COVID-19 cases) estimated the proportion of asymptomatic COVID-19 carriers at 15% (95% CI 12–18%). The transmission rates ranged from 0 to 2.2% for aymptomatic cases compared to 0.8–15.4% among symptomatic ones (102). Lau et al. estimated the presymptomatic transmission proportion to be 44% (95% CI, 30–57%) with infectiousness peaking between 2 days before and 1 day after symptoms onset (103). While a study carried out in Singapore found that around 6.4 % of the secondary infections are caused by the pre-symptomatic patients (104).

ROLE OF METEOROLOGICAL FACTORS DURING SARS-CoV-2 TRANSMISSION

The seasonal differences between the Southern and Northern hemispheres might have played a role in the spread of SARS-CoV-2. Early in the pandemic, Northern hemisphere countries with cold climates appeared to be the most vulnerable to COVID-19 transmission, while tropical regions and those in the Southern hemisphere seemed to be the least affected. Initial studies suggested a potential role for meterological factors in the spread of SARS-CoV-2. Sajadi et al. found more virus spread in areas with an average temperature of 5–11°C and absolute humidity of 4–7 g/m^3, suggesting a potentially seasonal behavior (105). Another study found that around 90% of the cases were reported in countries with temperatures maxima below 17°C and absolute humidity of 3–9 g/m^3. The study

suggested that the summer season might reduce the impact of COVID-19 pandemic in those countries as the temperatures rise (106). Another study concluded that SARS-CoV-2 transmits more easily in countries with relatively cool conditions and that transmission is reduced in sites with high temperatures and high relative humidity (107). Chen et al. reported that the optimal temperature for virus spread was found to be at 8°C and humidity between 60 and 90%. The authors suggested that the weather plays a key role in the transmission of SARS-CoV-2 around the world (108).

The association between the daily incidence of COVID-19 cases and climatic conditions in mainland China was examined between January 20 and February 29, 2020. Using modified susceptible-exposed-infectious-recovered (M-SEIR) model, Shi et al. found that COVID-19 transmission rate decreased at higher temperatures (109). However, another study conducted during the same period in China concluded that the increase in humidity and temperature alone would not reduce the virus spread if the public health interventions have not been strictly implemented (110). Similarly, a prospective cohort study done on 144 different areas other than China, South Korea, Iran, and Italy, found that it is the strict interventions that are strongly associated with the decrease in virus transmission but not latitude and temperature (111). Nonetheless, the early lockdowns in some countries and variable public interventions taken by various countries hindered the ability of scientists to study the association between climate and virus transmission. The aforementioned studies are also being challenged by the fact that many countries in the Northern hemisphere are witnessing a second wave of COVID-19 despite entering the summer season.

CONCLUSION: WILL COVID-19 BECOME SEASONAL?

The basic reproduction number (R0) is the number of secondary cases resulting from a primary case in a susceptible population and is an important indicator to predict the spread of a virus. For a virus to follow a seasonal pattern, and thus wane in summer, its effective R0 should drop below 1 (112). For SARS-CoV, the R0 is estimated between 2 and 3 (112) and in some estimates as high as 5.7 (113). As discussed above, several factors in the summer might reduce the effective R0 of respiratory viruses including the effect of warm tempertures and humidity on the stability of the virus and susceptibility of the host as well as behavior of the population such as indoor crowding. For seasonal influenza virus, its R0 is estimated to be 1.27 (114). Therefore, these aforementioned factors could easily drop the effective R0 to below 1 in summer halting the virus spread and resulting in the observed seasonal pattern of flu. The warm temperatures and humidity of the summer might impact the host immune response and thus its susceptibility to infection by SARS-CoV-2 similar to its effect on influenza (66). However, other factors including: (1) a much higher R0, (2) higher stability of SARS-CoV-2 (it can survive for up to 72 h on hard surfaces at temperatures between 21 and 23°C and in relative humidity of 40%) (83), and (3) a largely immunologically naïve population against SARS-CoV-2 compared to influenza make it unlikely for the R0 to drop in summer enough to halt the spread of SARS-CoV-2. Therefore, without public health interventions, SARS-CoV-2 will continue to spread in summer as witnessed in many countries around the world. Nonetheless, as the population herd immunity is attained through natural infections and/or vaccinations then the effective R0 is expected to drop substantially making the virus more prone to seasonal fluctuations.

AUTHOR CONTRIBUTIONS

HZ conceived the review idea and supervised the writing. AA developed the review outline and coordinated the drafting of the manuscript. AA, MA, MK, and GH wrote the manuscript. HY critically reviewed the manuscript. All authors revised and approved the final version of the manuscript.

REFERENCES

1. Zhou P, Yang X, Wang X-G, Hu B, Zhang L, Zhang W, et al. A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature*. (2020) 579:270–3. doi: 10.1038/s41586-020-2012-7
2. World Health Organization. *Coronavirus Disease (COVID-19) Situation Reports*. (2020) Available online at: https://www.who.int/emergencies/diseases/novel-coronavirus-2019/situation-reports (accessed May 13, 2020).
3. Perlman S, McIntosh K. Coronaviruses, including severe acute respiratory syndrome (SARS) and middle east respiratory syndrome (MERS). In: Bennett JE, Dolin R, Blaser MJ editors. *Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases*. Philadelphia, PA: Elsevier (2015) p. 2073–80.
4. Docea AO, Tsatsakis A, Albulescu D, Cristea O, Zlatian O, Vinceti M, et al. A new threat from an old enemy: re-emergence of coronavirus (review). *Int J Mol Med*. (2020) 45:1631–43. doi: 10.3892/ijmm.2020.4555
5. Fehr AR, Perlman S. Coronaviruses: an overview of their replication and pathogenesis. *Methods Mol Biol*. (2015) 1282:1–23. doi: 10.1007/978-1-4939-2438-7_1
6. World Health Organization. *World Health Organization | SARS (Severe Acute Respiratory Syndrome)*. WHO (2004). Available online at: https://www.who.int/ith/diseases/sars/en/ (accessed March 24, 2020).
7. World Health Organization. *World Health Organization EMRO | MERS Outbreaks | MERS-CoV | Health Topics*. (2020) Available online at: http://www.emro.who.int/health-topics/mers-cov/mers-outbreaks.html (accessed March 26, 2020).
8. Peiris JSM, Yuen KY, Osterhaus ADME, Stöhr K. The severe acute respiratory syndrome. *N Engl J Med*. (2003) 349:2431–41. doi: 10.1056/NEJMra032498
9. Zaki AM, van Boheemen S, Bestebroer TM, Osterhaus ADME, Fouchier RAM. Isolation of a novel coronavirus from a man with pneumonia in Saudi Arabia. *N Engl J Med*. (2012) 367:1814–20. doi: 10.1056/NEJMoa1211721
10. Memish ZA, Perlman S, Van Kerkhove MD, Zumla A. Middle east respiratory syndrome. *Lancet*. (2020) 395:1063–77. doi: 10.1016/S0140-6736(19)33221-0
11. World Health Organization. *Report of the WHO-China Joint Mission on Coronavirus Disease 2019 (COVID-19)*. (2020) Available online at: https://www.who.int/publications-detail/report-of-the-who-china-joint-mission-on-coronavirus-disease-2019-(covid-19) (accessed May 28, 2020).
12. World Health Organization. *Coronavirus Disease (COVID-19) Situation Report-10*. (2020) Available online at: https://www.who.int/emergencies/diseases/novel-coronavirus-2019/situation-reports (accessed May 13, 2020).
13. World Health Organization. *WHO Announces COVID-19 Outbreak a Pandemic*. (2020) Available online at: http://www.euro.who.int/en/health-

topics/health-emergencies/coronavirus-covid-19/news/news/2020/3/who-announces-covid-19-outbreak-a-pandemic (accessed March 31, 2020).
14. World Health Organization. *Coronavirus Disease (COVID-19) Situation Report-209*. (2020) Available online at: https://www.who.int/emergencies/diseases/novel-coronavirus-2019/situation-reports (accessed May 28, 2020).
15. AlTakarli NS. China's response to the COVID-19 outbreak: a model for epidemic preparedness and management. *DMJ.* (2020) 3:44–9. doi: 10.1159/000508448
16. Dickens BL, Koo JR, Lim JT, Park M, Quaye S, Sun H, et al. Modelling lockdown and exit strategies for COVID-19 in Singapore. *Lancet Region Health Western Pac.* (2020) 1:100004. doi: 10.1016/j.lanwpc.2020.100004
17. Kim J-H, An Julia A-R, Min P-k, Bitton A, Gawande AA. How South Korea responded to the Covid-19 outbreak in Daegu. *NEJM Catalyst.* (2020) 1. doi: 10.1056/CAT.20.0159
18. Martinez ME. The calendar of epidemics: seasonal cycles of infectious diseases. *PLoS Pathog.* (2018) 14:e1007327. doi: 10.1371/journal.ppat.1007327
19. Moriyama M, Hugentobler WJ, Iwasaki A. Seasonality of respiratory viral infections. *Annu Rev Virol.* (2020) 7. doi: 10.1146/annurev-virology-012420-022445
20. Price RHM, Graham C, Ramalingam S. Association between viral seasonality and meteorological factors. *Sci Rep.* (2019) 9:1–11. doi: 10.1038/s41598-018-37481-y
21. Lam TT, Tang JW, Lai FY, Zaraket H, Dbaibo G, Bialasiewicz S, et al. Comparative global epidemiology of influenza, respiratory syncytial and parainfluenza viruses, 2010–2015. *J Infect.* (2019) 79:373–82. doi: 10.1016/j.jinf.2019.07.008
22. Shek LP-C, Lee B-W. Epidemiology and seasonality of respiratory tract virus infections in the tropics. *Paediatr Respir Rev.* (2003) 4:105–11. doi: 10.1016/s1526-0542(03)00024-1
23. Jacobs SE, Lamson DM, George KS, Walsh TJ. Human rhinoviruses. *Clin Microbiol Rev.* (2013) 26:135–62. doi: 10.1128/CMR.00077-12
24. Dela Cruz CS, Pasnick S, Gross JE, Keller J, Carlos WG, Cao B, et al. Adenovirus infection and outbreaks: what you need to know. *Am J Respir Crit Care Med.* (2019) 199:P13–4. doi: 10.1164/rccm.1997P13
25. Isaacs D, Flowers D, Clarke JR, Valman HB, MacNaughton MR. Epidemiology of coronavirus respiratory infections. *Arch Dis Child.* (1983) 58:500–3.
26. Kaye HS, Marsh HB, Dowdle WR. Seroepidemiologic survey of coronavirus (strain OC 43) related infections in a children's population. *Am J Epidemiol.* (1971) 94:43–9. doi: 10.1093/oxfordjournals.aje.a121293
27. Hendley JO, Fishburne HB, Gwaltney JM. Coronavirus infections in working adults. Eight-year study with 229 E and OC 43. *Am Rev Respir Dis.* (1972) 105:805–11. doi: 10.1164/arrd.1972.105.5.805
28. Moës E, Vijgen L, Keyaerts E, Zlateva K, Li S, Maes P, et al. A novel pancoronavirus RT-PCR assay: frequent detection of human coronavirus NL63 in children hospitalized with respiratory tract infections in Belgium. *BMC Infect Dis.* (2005) 5:6. doi: 10.1186/1471-2334-5-6
29. Vabret A, Mourez T, Dina J, van der Hoek L, Gouarin S, Petitjean J, et al. Human coronavirus NL63, France. *Emerg Infect Dis.* (2005) 11:1225–9. doi: 10.3201/eid1108.050110
30. Suzuki A, Okamoto M, Ohmi A, Watanabe O, Miyabayashi S, Nishimura H. Detection of human coronavirus-NL63 in children in Japan. *Pediatr Infect Dis J.* (2005) 24:645–6. doi: 10.1097/01.inf.0000168846.71517.ee
31. Ebihara T, Endo R, Ma X, Ishiguro N, Kikuta H. Detection of human coronavirus NL63 in young children with bronchiolitis. *J Med Virol.* (2005) 75:463–5. doi: 10.1002/jmv.20289
32. Bastien N, Anderson K, Hart L, Van Caeseele P, Brandt K, Milley D, et al. Human coronavirus NL63 infection in Canada. *J Infect Dis.* (2005) 191:503–6. doi: 10.1086/426869
33. van der Hoek L, Sure K, Ihorst G, Stang A, Pyrc K, Jebbink MF, et al. Croup is associated with the novel coronavirus NL63. *PLoS Med.* (2005) 2:e240. doi: 10.1371/journal.pmed.0020240
34. Pierangeli A, Gentile M, Di Marco P, Pagnotti P, Scagnolari C, Trombetti S, et al. Detection and typing by molecular techniques of respiratory viruses in children hospitalized for acute respiratory infection in Rome, Italy. *J Med Virol.* (2007) 79:463–8. doi: 10.1002/jmv.20832
35. Kaplan NM, Dove W, Abd-Eldayem SA, Abu-Zeid AF, Shamoon HE, Hart CA. Molecular epidemiology and disease severity of respiratory syncytial virus in relation to other potential pathogens in children hospitalized with acute respiratory infection in Jordan. *J Med Virol.* (2008) 80:168–74. doi: 10.1002/jmv.21067
36. Esper F, Weibel C, Ferguson D, Landry ML, Kahn JS. Coronavirus HKU1 Infection in the United States. *Emerg Infect Dis.* (2006) 12:775–9. doi: 10.3201/eid1205.051316
37. Cui L-J, Zhang C, Zhang T, Lu R-J, Xie Z-D, Zhang L-L, et al. Human coronaviruses HCoV-NL63 and HCoV-HKU1 in hospitalized children with acute respiratory infections in Beijing, China. *Adv Virol.* (2011) 2011:129134. doi: 10.1155/2011/129134
38. Zeng Z-Q, Chen D-H, Tan W-P, Qiu S-Y, Xu D, Liang H-X, et al. Epidemiology and clinical characteristics of human coronaviruses OC43, 229E, NL63, and HKU1: a study of hospitalized children with acute respiratory tract infection in Guangzhou, China. *Eur J Clin Microbiol Infect Dis.* (2018) 37:363–9. doi: 10.1007/s10096-017-3144-z
39. Chiu SS, Chan KH, Chu KW, Kwan SW, Guan Y, Poon LLM, et al. Human coronavirus NL63 infection and other coronavirus infections in children hospitalized with acute respiratory disease in Hong Kong, China. *Clin Infect Dis.* (2005) 40:1721–9. doi: 10.1086/430301
40. Dare RK, Fry AM, Chittaganpitch M, Sawanpanyalert P, Olsen SJ, Erdman DD. Human coronavirus infections in rural Thailand: a comprehensive study using real-time reverse-transcription polymerase chain reaction assays. *J Infect Dis.* (2007) 196:1321–28. doi: 10.1086/521308
41. Arden KE, Nissen MD, Sloots TP, Mackay IM. New human coronavirus, HCoV-NL63, associated with severe lower respiratory tract disease in Australia. *J Med Virol.* (2005) 75:455–62. doi: 10.1002/jmv.20288
42. Smuts H. Human coronavirus NL63 infections in infants hospitalised with acute respiratory tract infections in South Africa. *Influenza Other Respir Viruses.* (2008) 2:135–8. doi: 10.1111/j.1750-2659.2008.00049.x
43. Owusu M, Annan A, Corman VM, Larbi R, Anti P, Drexler JF, et al. Human coronaviruses associated with upper respiratory tract infections in three rural areas of Ghana. *PLoS ONE.* (2014) 9:e99782. doi: 10.1371/journal.pone.0099782
44. Killerby ME, Biggs HM, Midgley CM, Gerber SI, Watson JT. Middle east respiratory syndrome coronavirus transmission. *Emerg Infect Dis J.* (2020) 26:191–8. doi: 10.3201/eid2602.190697
45. Dudas G, Carvalho LM, Rambaut A, Bedford T. MERS-CoV spillover at the camel-human interface. *eLife.* (2018) 7:e31257. doi: 10.7554/eLife.31257
46. Nassar MS, Bakhrebah MA, Meo SA, Alsuabeyl MS, Zaher WA. Global seasonal occurrence of middle east respiratory syndrome coronavirus (MERS-CoV) infection. *Eur Rev Med Pharmacol Sci.* (2018) 22:3913–8. doi: 10.26355/eurrev_201806_15276
47. Gardner EG, Kelton D, Poljak Z, Van Kerkhove M, von Dobschuetz S, Greer AL. A case-crossover analysis of the impact of weather on primary cases of Middle East respiratory syndrome. *BMC Infect Dis.* (2019) 19:113. doi: 10.1186/s12879-019-3729-5
48. Gautret P, Benkouiten S, Al-Tawfiq JA, Memish ZA. Hajj-associated viral respiratory infections: a systematic review. *Travel Med Infect Dis.* (2016) 14:92. doi: 10.1016/j.tmaid.2015.12.008
49. Ahmed QA, Arabi YM, Memish ZA. Health risks at the Hajj. *Lancet.* (2006) 367:1008–15. doi: 10.1016/S0140-6736(06)68429-8
50. Davis RE, Dougherty E, McArthur C, Huang QS, Baker MG. Cold, dry air is associated with influenza and pneumonia mortality in Auckland, New Zealand. *Influenza Other Respir Viruses.* (2016) 10:310–3. doi: 10.1111/irv.12369
51. Mäkinen TM, Juvonen R, Jokelainen J, Harju TH, Peitso A, Bloigu A, et al. Cold temperature and low humidity are associated with increased occurrence of respiratory tract infections. *Respir Med.* (2009) 103:456–62. doi: 10.1016/j.rmed.2008.09.011
52. Harper GJ. Airborne micro-organisms: survival tests with four viruses. *J Hyg.* (1961) 59:479–86. doi: 10.1017/s0022172400039176
53. Polozov IV, Bezrukov L, Gawrisch K, Zimmerberg J. Progressive ordering with decreasing temperature of the phospholipids of influenza virus. *Nat Chem Biol.* (2008) 4:248–55. doi: 10.1038/nchembio.77

54. Paynter S. Humidity and respiratory virus transmission in tropical and temperate settings. *Epidemiol Infect.* (2015) 143:1110–8. doi: 10.1017/S0950268814002702
55. Irwin CK, Yoon KJ, Wang C, Hoff SJ, Zimmerman JJ, Denagamage T, et al. Using the systematic review methodology to evaluate factors that influence the persistence of influenza virus in environmental matrices. *Appl Environ Microbiol.* (2011) 77:1049–60. doi: 10.1128/AEM.02733-09
56. Lowen A, Palese P. Transmission of influenza virus in temperate zones is predominantly by aerosol, in the tropics by contact. *PLoS Curr.* (2009) 1:RRN1002. doi: 10.1371/currents.RRN1002
57. Lowen AC, Mubareka S, Steel J, Palese P. Influenza virus transmission is dependent on relative humidity and temperature. *PLoS Pathog.* (2007) 3:1470–6. doi: 10.1371/journal.ppat.0030151
58. Tellier R. Review of aerosol transmission of influenza A virus. *Emerg Infect Dis.* (2006) 12:1657–62. doi: 10.3201/eid1211.060426
59. Yang W, Elankumaran S, Marr LC. Relationship between humidity and influenza A viability in droplets and implications for influenza's seasonality. *PLoS ONE.* (2012) 7:e46789. doi: 10.1371/journal.pone.0046789
60. Lytle CD, Sagripanti J-L. Predicted inactivation of viruses of relevance to biodefense by solar radiation. *J Virol.* (2005) 79:14244–52. doi: 10.1128/JVI.79.22.14244-14252.2005
61. Rauth AM. The physical state of viral nucleic acid and the sensitivity of viruses to ultraviolet light. *Biophys J.* (1965) 5:257–73. doi: 10.1016/s0006-3495(65)86715-7
62. Darnell MER, Taylor DR. Evaluation of inactivation methods for severe acute respiratory syndrome coronavirus in noncellular blood products. *Transfusion.* (2006) 46:1770–7. doi: 10.1111/j.1537-2995.2006.00976.x
63. Eccles R, Wilkinson JE. Exposure to cold and acute upper respiratory tract infection. *Rhinology.* (2015) 53:99–106. doi: 10.4193/Rhin14.239
64. Castellani JW, Brenner MIK, Rhind SG. Cold exposure: human immune responses and intracellular cytokine expression. *Med Sci Sports Exerc.* (2002) 34:2013–20. doi: 10.1097/00005768-200212000-00023
65. Shephard RJ, Shek PN. Cold exposure and immune function. *Can J Physiol Pharmacol.* (1998) 76:828–36. doi: 10.1139/cjpp-76-9-828
66. Eccles R. An explanation for the seasonality of acute upper respiratory tract viral infections. *Acta Otolaryngol.* (2002) 122:183–91. doi: 10.1080/00016480252814207
67. Kudo E, Song E, Yockey L, Rakib T, Wong P, Homer R, et al. Low ambient humidity impairs barrier function and innate resistance against influenza infection. *Proc Natl Acad Sci USA.* (2019) 116:201902840. doi: 10.1073/pnas.1902840116
68. Donaldson GC, Seemungal T, Jeffries DJ, Wedzicha JA. Effect of temperature on lung function and symptoms in chronic obstructive pulmonary disease. *Eur Respir J.* (1999) 13:844–9. doi: 10.1034/j.1399-3003.1999.13d25.x
69. Foxman EF, Storer JA, Fitzgerald ME, Wasik BR, Hou L, Zhao H, et al. Temperature-dependent innate defense against the common cold virus limits viral replication at warm temperature in mouse airway cells. *Proc Natl Acad Sci USA.* (2015) 112:827–32. doi: 10.1073/pnas.1411030112
70. Bustamante-Marin XM, Ostrowski LE. Cilia and mucociliary clearance. *Cold Spring Harb Perspect Biol.* (2017) 9:a028241. doi: 10.1101/cshperspect.a028241
71. Even-Tzur N, Zaretsky U, Grinberg O, Davidovich T, Kloog Y, Wolf M, et al. Climate chamber for environmentally controlled laboratory airflow experiments. *Technol Health Care.* (2010) 18:157–63. doi: 10.3233/THC-2010-0577
72. Baetjer AM. Effect of ambient temperature and vapor pressure on cilia-mucus clearance rate. *J Appl Physiol.* (1967) 23:498–504. doi: 10.1152/jappl.1967.23.4.498
73. Clary-Meinesz CF, Cosson J, Huitorel P, Blaive B. Temperature effect on the ciliary beat frequency of human nasal and tracheal ciliated cells. *Biol Cell.* (1992) 76:335–8. doi: 10.1016/0248-4900(92)90436-5
74. Barbet JP, Chauveau M, Labbé S, Lockhart A. Breathing dry air causes acute epithelial damage and inflammation of the guinea pig trachea. *J Appl Physiol.* (1988) 64:1851–7. doi: 10.1152/jappl.1988.64.5.1851
75. Salman H, Bergman M, Bessler H, Alexandrova S, Beilin B, Djaldetti M. Hypothermia affects the phagocytic activity of rat peritoneal macrophages. *Acta Physiol Scand.* (2000) 168:431–6. doi: 10.1046/j.1365-201x.2000.00686.x
76. Schwalfenberg GK. A review of the critical role of vitamin D in the functioning of the immune system and the clinical implications of vitamin D deficiency. *Mol Nutr Food Res.* (2011) 55:96–108. doi: 10.1002/mnfr.201000174
77. Berry DJ, Hesketh K, Power C, Hyppönen E. Vitamin D status has a linear association with seasonal infections and lung function in British adults. *Br J Nutr.* (2011) 106:1433–40. doi: 10.1017/S0007114511001991
78. Martineau AR, Jolliffe DA, Hooper RL, Greenberg L, Aloia JF, Bergman P, et al. Vitamin D supplementation to prevent acute respiratory tract infections: systematic review and meta-analysis of individual participant data. *BMJ.* (2017) 356:i6583. doi: 10.1136/bmj.i6583
79. Moan J, Dahlback A, Ma L, Juzeniene A. Influenza, solar radiation and vitamin D. *Dermatoendocrinol.* (2009) 1:307–9. doi: 10.4161/derm.1.6.11357
80. Alipio M. *Vitamin D Supplementation Could Possibly Improve Clinical Outcomes of Patients Infected With Coronavirus-2019 (COVID-19).* Rochester, NY: Social Science Research Network (2020). Available online at: https://papers.ssrn.com/abstract=3571484 (accessed May 28, 2020).
81. Shaman J, Kohn M. Absolute humidity modulates influenza survival, transmission, and seasonality. *Proc Natl Acad Sci USA.* (2009) 106:3243–8. doi: 10.1073/pnas.0806852106
82. World Health Organization. *Transmission of SARS-CoV-2: Implications for Infection Prevention Precautions*. Available online at: https://www.who.int/news-room/commentaries/detail/transmission-of-sars-cov-2-implications-for-infection-prevention-precautions (accessed August 8, 2020).
83. van Doremalen N, Bushmaker T, Morris DH, Holbrook MG, Gamble A, Williamson BN, et al. Aerosol and surface stability of SARS-CoV-2 as compared with SARS-CoV-1. *N Engl J Med.* (2020) 382:1564–7. doi: 10.1056/NEJMc2004973
84. Chia PY, Coleman KK, Tan YK, Ong SWX, Gum M, Lau SK, et al. Detection of air and surface contamination by SARS-CoV-2 in hospital rooms of infected patients. *Nat Commun.* (2020) 11:2800. doi: 10.1038/s41467-020-16670-2
85. Matson MJ, Yinda CK, Seifert SN, Bushmaker T, Fischer RJ, Doremalen N van, et al. Early release - effect of environmental conditions on SARS-CoV-2 stability in human nasal mucus and sputum. *Emerg Infect Dis J.* (2020) 26:2276–8. doi: 10.3201/eid2609.202267
86. Pastorino B, Touret F, Gilles M, de Lamballerie X, Charrel RN. Prolonged Infectivity of SARS-CoV-2 in Fomites. *Emerg Infect Dis.* (2020) 26:2256–7. doi: 10.3201/eid2609.201788
87. Morawska L, Cao J. Airborne transmission of SARS-CoV-2: the world should face the reality. *Environ Int.* (2020) 139:105730. doi: 10.1016/j.envint.2020.105730
88. Liu Y, Ning Z, Chen Y, Guo M, Liu Y, Gali NK, et al. Aerodynamic analysis of SARS-CoV-2 in two Wuhan hospitals. *Nature.* (2020) 582:1–6. doi: 10.1038/s41586-020-2271-3
89. Santarpia JL, Rivera DN, Herrera VL, Morwitzer MJ, Creager HM, Santarpia GW, et al. Aerosol and surface contamination of SARS-CoV-2 observed in quarantine and isolation care. *Sci Rep.* (2020) 10:12732. doi: 10.1038/s41598-020-69286-3
90. Lednicky JA, Lauzardo M, Fan ZH, Jutla AS, Tilly TB, Gangwar M, et al. Viable SARS-CoV-2 in the air of a hospital room with COVID-19 patients. *medRxiv.* (2020). doi: 10.1101/2020.08.03.20167395
91. Wang W, Xu Y, Gao R, Lu R, Han K, Wu G, et al. Detection of SARS-CoV-2 in different types of clinical specimens. *JAMA.* (2020) 323:1843–4. doi: 10.1001/jama.2020.3786
92. Wu Y, Guo C, Tang L, Hong Z, Zhou J, Dong X, et al. Prolonged presence of SARS-CoV-2 viral RNA in faecal samples. *Lancet Gastroenterol Hepatol.* (2020) 5:434–5. doi: 10.1016/S2468-1253(20)30083-2
93. Zheng S, Fan J, Yu F, Feng B, Lou B, Zou Q, et al. Viral load dynamics and disease severity in patients infected with SARS-CoV-2 in Zhejiang province, China, January-March 2020: retrospective cohort study. *BMJ.* (2020) 369:m1443. doi: 10.1136/bmj.m1443
94. Sun J, Zhu A, Li H, Zheng K, Zhuang Z, Chen Z, et al. Isolation of infectious SARS-CoV-2 from urine of a COVID-19 patient. *Emerg Microbes Infect.* (2020) 9:991–3. doi: 10.1080/22221751.2020.1760144

95. Xiao F, Sun J, Xu Y, Li F, Huang X, Li H, et al. Infectious SARS-CoV-2 in Feces of patient with severe COVID-19. *Emerg Infect Dis.* (2020) 26:1920–2. doi: 10.3201/eid2608.200681
96. Chang L, Zhao L, Gong H, Wang L, Wang L. Severe acute respiratory syndrome coronavirus 2 RNA detected in blood donations. *Emerg Infect Dis.* (2020) 26:1631–3. doi: 10.3201/eid2607.200839
97. World Health Organization. *Breastfeeding and COVID-19*. Available online at: https://www.who.int/news-room/commentaries/detail/breastfeeding-and-covid-19 (accessed August 9, 2020).
98. Mizumoto K, Kagaya K, Zarebski A, Chowell G. Estimating the asymptomatic proportion of coronavirus disease 2019 (COVID-19) cases on board the Diamond Princess cruise ship, Yokohama, Japan, 2020. *Euro Surveill.* (2020) 25:2000180. doi: 10.2807/1560-7917.ES.2020.25.10.2000180
99. Arons MM, Hatfield KM, Reddy SC, Kimball A, James A, Jacobs JR, et al. Presymptomatic SARS-CoV-2 infections and transmission in a skilled nursing facility. *N Engl J Med.* (2020) 382:2081–90. doi: 10.1056/NEJMoa2008457
100. Tong Z-D, Tang A, Li K-F, Li P, Wang H-L, Yi J-P, et al. Potential presymptomatic transmission of SARS-CoV-2, Zhejiang Province, China. *Emerg Infect Dis J.* (2020) 26:1052–4. doi: 10.3201/eid2605.200198
101. Savvides C, Siegel R. Asymptomatic and presymptomatic transmission of SARS-CoV-2: a systematic review. *medRxiv. [Preprint].* (2020). doi: 10.1101/2020.06.11.20129072
102. Byambasuren O, Cardona M, Bell K, Clark J, McLaws M-L, Glasziou P. Estimating the extent of asymptomatic COVID-19 and its potential for community transmission: systematic review and meta-analysis. *medRxiv. [Preprint].* (2020). doi: 10.1101/2020.05.10.20097543
103. He X, Lau EHY, Wu P, Deng X, Wang J, Hao X, et al. Temporal dynamics in viral shedding and transmissibility of COVID-19. *Nat Med.* (2020) 26:672–5. doi: 10.1038/s41591-020-0869-5
104. Wei WE. Presymptomatic transmission of SARS-CoV-2—Singapore, January 23–March 16, 2020. *MMWR Morb Mortal Wkly Rep.* (2020) 69:411–5. doi: 10.15585/mmwr.mm6914e1
105. Sajadi M, Habibzadeh P, Vintzileos A, Shokouhi S, Miralles-Wilhelm F, Amoroso A. Temperature and latitude analysis to predict potential spread and seasonality for COVID-19. *SSRN Electr J.* (2020). doi: 10.2139/ssrn.3550308
106. Bukhari Q, Jameel Y. *Will Coronavirus Pandemic Diminish by Summer?* Rochester, NY: Social Science Research Network (2020).
107. Wang J, Tang K, Feng K, Lv W. High temperature and high humidity reduce the transmission of COVID-19. *arXiv.* (2020). doi: 10.2139/ssrn.3551767
108. Chen B, Liang H, Yuan X, Hu Y, Xu M, Zhao Y, et al. Roles of meteorological conditions in COVID-19 transmission on a worldwide scale. *medRxiv. [Preprint].* (2020). doi: 10.1101/2020.03.16.20037168
109. Shi P, Dong Y, Yan H, Li X, Zhao C, Liu W, et al. The impact of temperature and absolute humidity on the coronavirus disease 2019 (COVID-19) outbreak - evidence from China. *medRxiv. [Preprint].* (2020). doi: 10.1101/2020.03.22.20038919
110. Luo W, Majumder M, Liu D, Poirier C, Mandl K, Lipsitch M, et al. The role of absolute humidity on transmission rates of the COVID-19 outbreak. *medRxiv. [Preprint].* (2020). doi: 10.1101/2020.02.12.20022467
111. Jüni P, Rothenbühler M, Bobos P, Thorpe KE, Costa BR da, Fisman DN, et al. Impact of climate and public health interventions on the COVID-19 pandemic: a prospective cohort study. *CMAJ.* (2020) 192:E566–73. doi: 10.1503/cmaj.200920
112. Petersen E, Koopmans M, Go U, Hamer DH, Petrosillo N, Castelli F, et al. Comparing SARS-CoV-2 with SARS-CoV and influenza pandemics. *Lancet Infect Dis.* (2020) 20:E238–44. doi: 10.1016/S1473-3099(20)30484-9
113. Sanche S, Lin YT, Xu C, Romero-Severson E, Hengartner N, Ke R. High contagiousness and rapid spread of severe acute respiratory syndrome coronavirus 2. *Emerg Infect Dis J.* (2020) 7:1470–7. doi: 10.3201/eid2607.200282
114. Biggerstaff M, Cauchemez S, Reed C, Gambhir M, Finelli L. Estimates of the reproduction number for seasonal, pandemic, and zoonotic influenza: a systematic review of the literature. *BMC Infect Dis.* (2014) 14:480. doi: 10.1186/1471-2334-14-480

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 15 September 2020
doi: 10.3389/fcimb.2020.548582

Analysis of Risk Factors for 24 Patients With COVID-19 Developing From Moderate to Severe Condition

Dianming Li[1], *Chuanmiao Liu*[2], *Jiahui Liu*[1], *Junfeng Hu*[1], *Yanli Yang*[3]* *and Yufu Zhou*[4]

[1] Department of Respiratory and Critical Care Medicine, The First Affiliated Hospital of Bengbu Medical College, Bengbu, China, [2] Department of Infectious Diseases, The First Affiliated Hospital of Bengbu Medical College, Bengbu, China, [3] Department of Hematology, The First Affiliated Hospital of Bengbu Medical College, Bengbu, China, [4] Department of Tumor Radiotherapy, The First Affiliated Hospital of Bengbu Medical College, Bengbu, China

Objective: The present study aimed at investigating the clinical risk factors for COVID-19 patients developing from moderate condition to severe condition, and providing reference for early intervention and prognosis.

Methods: We collected the clinical data of 24 patients with moderate-to-severe COVID-19 who were admitted to the isolation ward of the First Affiliated Hospital of Bengbu Medical College from January, 2020 to February 20, 2020, and evaluated the data of clinical characteristics, blood test results, inflammatory index, chest CT imaging characteristics, and antiviral treatment, comparing this with the clinical data of 41 patients with moderate condition in the same period. From this comparison we thus summarized the current knowledge of potential risk factors for COVID-19 patients developing from moderate to severe condition.

Results: (1) Clinical characteristics: The moderate-to-severe group and the moderate group in terms of combined common underlying diseases and respiratory frequency showed significant difference statistically (*t*-value were 13.32, 6.17, respectively, $P < 0.05$), while no significant difference between the two groups in gender, age, or clinical symptoms was statistically observed($P > 0.05$).

(2) Analysis of blood test results: The lymphocyte count and plasma albumin of the moderate-to-severe group were significantly lower than those of the moderate group (*t*-values were 4.16, 4.11, respectively, $P < 0.05$), and the blood glucose and urea of the moderate-to-severe group were significantly higher than those of the moderate group (*t*-value were 3.27, 4.19, respectively, $P < 0.05$). However, there was no significant difference in terms of white blood cell count (WBC), platelet count (PLT), and glutamic-pyruvic transaminase (GPT) ($P > 0.05$).

(3) Comparison of inflammatory indicators: The level of IL-6 and CRP of the moderate-to-severe group were significantly higher than those of the moderate group (*t*-values were 2.84, 4.88, respectively, $P < 0.05$).

(4) Imaging comparison: As for patients with moderate COVID-19, the imaging manifestations were the concurrence of ground-glass opacity, patchy shadow, and consolidation shadow in both lungs, diffuse ground-glass opacity in both lungs accompanied by air bronchogram, and large area consolidation of both lungs with

OPEN ACCESS

Edited by:
Slobodan Paessler,
The University of Texas Medical Branch at Galveston, United States

Reviewed by:
Brian Carey,
United States Army Medical Research Institute of Infectious Diseases (USAMRIID), United States
Sabrina John Moyo,
University of Bergen, Norway
Robert Cody Sharp,
University of Florida Health, United States

***Correspondence:**
Yanli Yang
yangyanli0702@126.com

Specialty section:
This article was submitted to Clinical Microbiology, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: *03 April 2020*
Accepted: *25 August 2020*
Published: *15 September 2020*

Citation:
Li D, Liu C, Liu J, Hu J, Yang Y and Zhou Y (2020) Analysis of Risk Factors for 24 Patients With COVID-19 Developing From Moderate to Severe Condition. Front. Cell. Infect. Microbiol. 10:548582. doi: 10.3389/fcimb.2020.548582

pulmonary interstitial changes. The possibility for these patients to develop into severe condition increased, and the differences were statistically significant ($t = 10.92, P < 0.05$). (5) Clinical antiviral treatment: There was no statistically significant difference in the combination of two or three antiviral drugs between the two groups ($\chi^2 = 0.05, P > 0.05$).

Conclusion: Current evidence suggested that the combination of common underlying diseases, respiratory frequency, lymphocyte count, blood glucose, albumin, urea level, inflammatory factors (CRP, IL-6), and imaging manifestations collectively contributed to the potential risk factors for the development of COVID-19 from moderate condition to severe condition. Particular attention should be paid to early detection and intervention during clinical work, which will be of vital significance to the ascent of the recovery rate as well as the reduction of mortality.

Keywords: COVID-19, moderate, severe, risk factors, IL-6

INTRODUCTION

Since December 2019, patients with novel coronavirus pneumonia have been detected in the city of Wuhan in Hubei province. With the rapid spread of the epidemic, additional cases have been found in other parts of China and abroad. The disease was officially named "2019 coronavirus disease" (COVID-19) by the Director-General of WHO, Tan Desai, on February 11th and a subsequent announcement by the National Center for Disease Control and Prevention declared the inclusion of COVID-19 in the national "class B" infectious diseases and the adoption of "class A" infectious disease prevention and control measures Novel coronavirus pneumonia diagnosis and treatment plan of People's Republic of China national health and Health Committee. Fundamental clinical and epidemiological studies on COVID-19 have been reported recently (Chan J. et al., 2020; Chen L. et al., 2020; Huang et al., 2020; Ren et al., 2020; Wang et al., 2020). With the efforts of positive and effective prevention and control measures throughout the country and the devotion from the vast majority of medical workers, the epidemic has been basically controlled. However, the number of cases has continued to grow dramatically among overseas countries, especially Italy, Spain, and others, accompanied by a relative high mortality rate. We found that some moderate patients tend to develop into severe condition in a short period of time, or even become critical, so it is very difficult to improve the level of treatment. The distinction for these patients in the early stage will be of great value to the improvement of diagnosis and treatment. In the study, we collected clinical data derived from 24 patients admitted to our hospital with COVID-19 developing from moderate to severe condition, compared this with the clinical data of 41 patients with moderate condition in the same period, and analyzed the potential risk factors for COVID-19 patients developing from moderate to severe condition.

PATIENTS AND METHODS

Patients

The study has been approved by the medical ethics committee of the First Affiliated Hospital of Bengbu Medical College, which conforms to the principles of the Declaration of Helsinki. Our hospital is the designated hospital for COVID-19 in Anhui Province, and one of the four intensive treatment bases for severe patients in the province. All the cases meet the COVID-19 diagnostic criteria (sixth edition): (1) epidemiological history: travel history or residential history in Wuhan and its surrounding areas or other communities with reported cases within 14 days before onset; history of contact with a novel coronavirus infected person (positive in nucleic acid test) within 14 days before onset; exposure to patients with fever or respiratory symptoms from Wuhan and its surrounding areas or other communities with reported cases within 14 days before onset; and a clustering onset of disease. (2) Clinical manifestations: fever and/or respiratory symptoms; the aforementioned imaging characteristics of COVID-19; and the total WBC is normal or decreased in the early stage, or the lymphocyte count is decreased. A suspected case can be diagnosed if the patient has any one of the characteristics of epidemiological histories and conforms to any two of the clinical manifestations. If the patient has no epidemiological history and conforms to three of the clinical manifestations, it can be diagnosed as a suspected case. The inclusion criteria for confirmed cases was: suspected cases with one of the following pieces of etiological evidence: positive nucleic acid of novel coronavirus was detected in Real-time fluorescence RT-PCR; or gene sequencing of the virus revealed a high homology with the novel coronavirus. We collected the clinical data of 24 patients with moderate-to-severe COVID-19 who were admitted to the isolation ward of the First Affiliated Hospital of Bengbu Medical College from January, 2020 to February 20, 2020, and evaluated the clinical characteristics, blood test results, inflammatory index, chest CT imaging characteristics, and antiviral treatment, comparing these with the clinical data of 41 patients with moderate condition in the same period, and summarized the potential risk factors for COVID-19 patients developing from moderate to severe condition.

Clinical Typing of Disease Severity

All confirmed patients were clinically classified according to the "diagnosis and treatment plan of novel coronavirus pneumonia"

TABLE 1 | Comparison of clinical characteristics between two groups.

General information		Moderate-to-severe Group ($N = 24$)	Moderate Group ($N = 41$)	χ^2/t	*P*-value
Gender	Male	15	20	1.15	>0.05
	Female	9	21		
Age	<40 yr	1	5	2.52	>0.05
	40–50 yr	4	11		
	51–60 yr	12	15		
	≥60 yr	7	10		
Combined underlying diseases	1	13	36	13.32	<0.05
	≥2	11	5		
Clinical symptoms	Fever	21	41	3.41	>0.05
	Cough/sputum	8	12		
	chest distress and dyspnea	3	1		
	others	0	1		
Main physical signs	<24 times /min	16	36	6.13	<0.05
	≥24 times /min	8	5		

TABLE 2 | Comparison of hematological indexes between the two groups.

Hematological index	Moderate-to-severe Group ($N = 24$)	Moderate Group ($N = 41$)	*t*	*P*-value
WBC ($\times 10^9$/L)	6.34 ± 3.68	6.42 ± 3.53	0.09	>0.05
Lymphocyte count ($\times 10^9$/L)	0.98 ± 0.53	1.56 ± 0.55	4.16	<0.05
PLT ($\times 10^9$/L)	237.58 ± 125.53	257.68 ± 84.12	0.77	>0.05
Plasma albumin (g/L)	35.03 ± 5.92	40.01 ± 3.85	4.11	<0.05
ALT (U/L)	42.88 ± 41.83	36.73 ± 34.71	0.63	>0.05
Blood glucose (mmol/L)	8.84 ± 3.88	6.51 ± 1.86	3.27	<0.05
Urea (umol/L)	5.97 ± 2.96	3.74 ± 1.31	4.19	<0.05

TABLE 3 | Comparison of inflammatory indicators between the two groups.

Inflammatory indicators	Moderate-to-severe Group ($N = 24$)	Moderate Group ($N = 41$)	*t*	*P*-value
IL-6 (pg/ml)	9.88 ± 4.59	6.69 ± 4.23	2.84	<0.01
CRP (mg/L)	47.88 ± 16.63	28.35 ± 14.91	4.88	<0.01

at admission. Moderate condition was classified as a patient with fever and respiratory symptoms with whom manifestations of pneumonia can be found in imaging findings. Severe condition was classified based on any of the following: respiratory distress, RR ≥ 30 times/min; under resting state, oxygen saturation ≤93%; and arterial partial oxygen pressure (PaO_2)/oxygen concentration (FiO_2) ≤300 mmHg (1 mmHg = 0.133 kpa). Patients with lesion progression more than 50% shown by pulmonary imaging within 24–48 h were managed under severe care Novel coronavirus pneumonia diagnosis and treatment plan of People's Republic of China national health and Health Committee.

Test Method

In the morning of the second day after admission, 2 ml of the fasting venous blood was taken and sent to the laboratory to check the blood routine and blood biochemistry; automatic blood cell analyzer and blood biochemical analyzer were used for detection. The serum inflammatory factor IL-6 was detected by enzyme-linked immunosorbent assay (ELISA). The operation was carried out in strict accordance with the manual. The kit was purchased from eBioscience company (EPX650-16500-901).

Statistical Analysis

SPSS 19.0 was used. The counting data of normally distributed measurements was expressed by $\bar{x} \pm s$, *t*-test was conducted, and the measurement data in percentage were tested by χ^2/t. $P < 0.05$ was considered statistically significant.

RESULTS

Comparison of Clinical Characteristics

The general information of the 24 patients developing from moderate to severe condition on admission included: the median age, which was 56 years (21–83 years); 15 males (62.50%) and nine females (37.50%); and 13 patients (54.17%) with one or no common underlying diseases, and 11 patients (45.83%) with more than two kinds of common underlying diseases, such as hypertension, coronary heart disease, diabetes, etc. The 41 moderate patients were 30–78 years old, and the median age was 53 years old; there were 20 male patients (48.78%), and 21 female patients (51.22%); 36 patients (87.80%) had one or no common underlying diseases, and five patients (12.20%) had more than two kinds of common underlying diseases. Among the 24 confirmed patients, symptoms and signs included: 21 patients (87.50%) with fever, eight patients (33.33%) with cough and sputum, three patients (12.50%) with chest tightness and dyspnea, and no patients (0%) with other symptoms. The main physical signs included increased respiratory frequency (≥24 times /min) in eight patients (33.33%). Among the 41 confirmed cases, 41 patients (100%) had fever, 12 patients (29.27%) had cough and expectoration, one patient (2.44%) had chest distress and dyspnea, and one patient (2.44%) had other signs. The main signs included increased respiratory rate (≥24 times/min) in five patients (12.20%). By comparison, there were significant differences in combined common underlying diseases and respiratory frequency between the moderate group and the severe group (*t*-values were 13.32, 6.17, respectively, $P < 0.05$), while there were no significant differences in gender, age, or clinical symptoms between the two groups ($P > 0.05$) (**Table 1**).

Comparison of Blood Test Results

The comparison of hematological indexes on the second day after admission of the two groups of patients showed that the

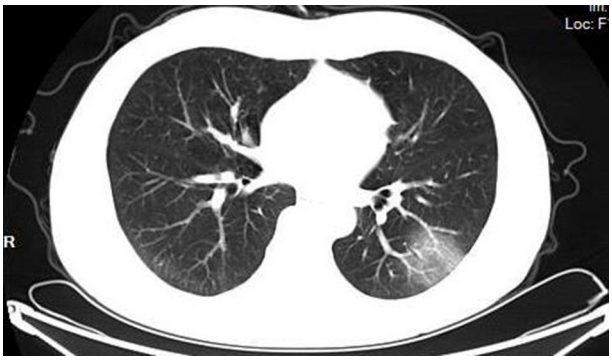


FIGURE 1 | Single ground-glass opacity.

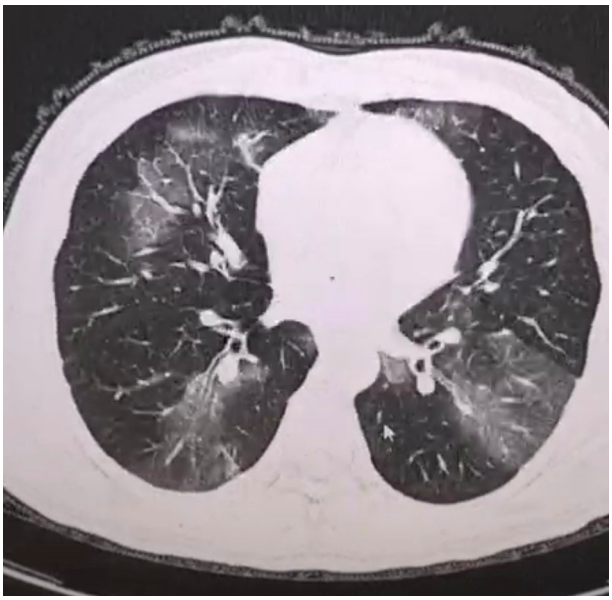


FIGURE 2 | Multiple external subpleural ground-glass opacity.

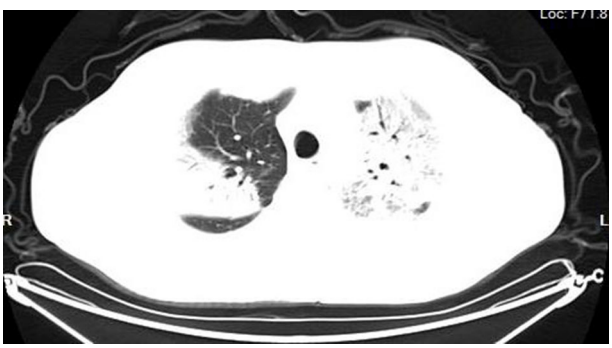


FIGURE 3 | Ground-glass opacity, patchy shadow, and consolidation shadow in both lungs.

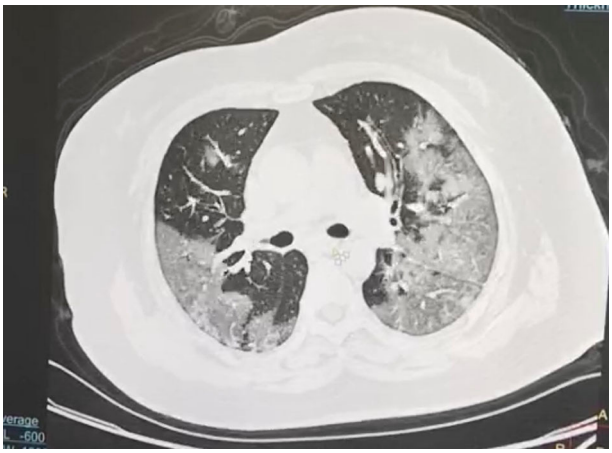


FIGURE 4 | Diffuse ground-glass opacity in both lungs accompanied by air bronchogram.

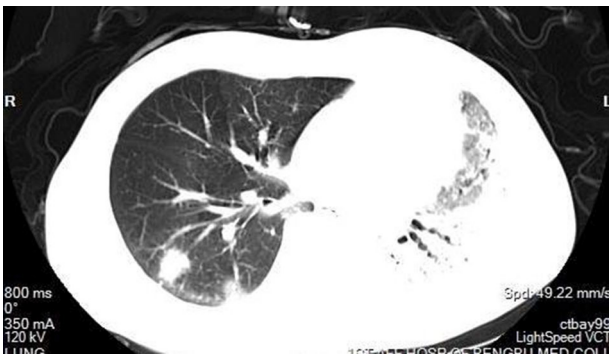


FIGURE 5 | Large area consolidation of both lungs with pulmonary interstitial changes.

lymphocyte count and plasma albumin in the moderate-to-severe group were significantly lower than that in the moderate group (t-values were 4.16, 4.11, respectively, $P < 0.05$). The levels of blood glucose and urea in the moderate-to-severe group were significantly higher than the moderate group (t-values were 3.27, 4.19, respectively, $P < 0.05$). However, there was no significant difference in terms of white blood cell count (WBC), platelet count (PLT), or glutamic-pyruvic transaminase (GPT) ($P > 0.05$) (**Table 2**).

Comparison of Inflammatory Indicators

The levels of IL-6 and CRP of the moderate-to-severe group were significantly higher than those of the moderate group (t-values were 2.84, 4.88, respectively, $P < 0.05$) (**Table 3**).

Comparison of Imaging Manifestation

According to the CT imaging manifestation of 65 COVID-19 patients, the typical imaging signs were (**Figures 1–5**): (1) single ground-glass opacity (GGO); (2) multiple external subpleural ground-glass opacity; (3) concurrence of ground-glass opacity,

TABLE 4 | Comparison of imaging manifestation between the two groups.

Imaging manifestation	Moderate-to-severe Group (%)	Moderate Group (%)	*t*	*P*-value
Single ground-glass opacity	1 (4.17%)	8 (19.51%)	10.92	<0.05
Multiple external subpleural Ground-glass opacity	4 (16.67%)	17 (41.46%)		
Ground-glass opacity, patchy shadow and consolidation shadow in both lungs	11 (45.83%)	10 (24.39%)		
Diffuse ground-glass opacity in both lungs accompanied by air bronchogram	7 (29.16%)	6 (14.64%)		
Large area consolidation of both lungs with pulmonary interstitial changes	1 (4.17%)	0		

TABLE 5 | Comparison of antiviral treatment between the two groups.

Antiviral treatment	Moderate-to-severe Group	Moderate Group	χ^2	*P*-value
Combination of 2 antiviral treatment	20	35	0.05	>0.05
Combination of 3 antiviral treatment	4	6		

patchy shadow, and consolidation shadow in both lungs; (4) diffuse ground-glass opacity in both lungs accompanied by air bronchogram; and (5) large area consolidation of both lungs with pulmonary interstitial changes. Comparisons of the imaging manifestation between the two groups showed a statistically significant difference ($t = 10.92$, $P < 0.05$). In terms of the proportion of the latter three typical signs, the moderate-to-severe group was higher than the moderate group (**Table 4**).

Comparison of Antiviral Treatment

For clinical antiviral treatment of COVID-19, according to the current scheme, it is recommended to use abidol, klidge, interferon, chloroquine, ribavirin, etc. Two or three used in conjunction are recommended. The dosage, method, and course of treatment are in accordance with the national covid-19 treatment program. This study shows no statistically significant difference in the combination of two antiviral drugs or the combination of three antiviral drugs between the two groups ($\chi^2 = 0.05$, $P > 0.05$) (**Table 5**).

DISCUSSION

It can be suggested that some of the moderate patients can develop to the severe type, or even to the critical type, within a very short period of time from the clinical work, which makes the improvement of the remedy rate of clinical treatment more difficult. The detection of these patients from the early stage will be worthwhile to the improvement of diagnosis and treatment. This study analyzed the clinical data of 24 COVID-19 patients admitted to our hospital developing from moderate to severe condition, and compared this with the clinical data of 41 moderate patients in the same period; the results were consistent with the results of Guo et al. (2019). The most common underlying diseases are hypertension, diabetes, and coronary heart disease. This may be because the majority of patients are the elderly with a compromised immunity, so we should pay extra attention to elderly patients. However, further analysis of gender, age, and clinical symptoms revealed no significant correlation with the development from moderate to severe condition ($P > 0.05$), which might be attributed to the small number of cases. (2) Compared with the hematological indexes of the two groups after admission, the lymphocyte count and plasma albumin levels in the moderate-to-severe group were significantly lower than those in the moderate group (t-values were 4.16, 4.11, respectively, $P < 0.05$). The decrease of lymphocyte count indicates the possibility of immune impairment, and the decrease of albumin may contribute to the hepatic albumin synthesis disorder caused by the direct damage of the virus to hepatocytes. Some researchers (Guo et al., 2019) proposed to use lymphocyte count $<0.8 \times 10^9$/L as one of the indicators to predict the death risk model of viral pneumonia. The moderate-to-severe group was significantly higher than the moderate group in blood glucose and urea (t-values were 3.27, 4.19, respectively, $P < 0.05$), these patients with elevated blood glucose and urea are more likely to develop to severe type. The increase of blood glucose may be connected with the severity of patients or the existence of complicated diabetes, while the increase of BUN may be related to the severe inflammatory response and the hypermetabolism caused by fever in severe patients. However, there was no significant difference in contrast with WBC, PLT, and GBT ($P > 0.05$). It is inferred that moderate patients with a low lymphocyte count and low albumin on admission are more likely to develop to severe condition, which is consistent with the results of most clinical studies (Chen Y. et al., 2020), thus more efforts should be given to these cases. With great individual diversities, we also found that patients had different immune responses to the virus, leading to vast varieties in clinical symptoms, disease progression, and response to therapeutic drugs (Castrucci, 2018). Therefore, for the clinical progression of COVID-19, we should also consider the differences in individual inflammatory responses and search for some objective indicators to help accurately predict the clinical progression and cover the deficiency.

It is well-known that the immune function can enable the body to acquire the defense ability required to resist external infection and eliminate foreign microorganisms, thereby inhibiting the infection and restoring health. But everything has two sides. When the virus invades the body, if the immune system is overactivated or out of control, it will produce an extreme immune response, and release large amounts of cytokines, which in turn attack the host. This phenomenon is called "inflammatory storm." Numerous evidences have shown that cytokines and chemokines are significantly elevated in patients with severe infection and are considered to reflect the severity of the disease (La Gruta et al., 2007). Studies have

shown that the expression levels of serum IL-2R, IL-6, and other cytokines in patients with COVID-19 dramatically increased on average, particularly in the critical patients. Therefore, some scholars pointed out that peripheral blood IL-6 could be applied as a key factor to independently predict the progression of COVID-19. It can be speculated on the basis of that, as a blocking target, IL-6 might have potential clinical value in inhibiting the inflammatory response. Tocilizumab, namely human anti-human interleukin-6 receptor monoclonal antibody, suppresses the activity of IL-6 in peripheral blood to block or reduce the inflammatory response (Yang et al., 2020). With the detection of serum CRP and IL-6 in patients, we found that the levels of IL-6 and CRP in the moderate-to-severe group were significantly higher than that in the moderate group (t-values were 2.84, 4.88, respectively, $P < 0.05$), indicating the possibility of immunocompromise. The moderate patients with elevated CRP and IL-6 are more likely to develop into severe condition.

In order to understand the status of lung lesions in COVID-19 patients, we recommend chest HRCT to avoid misdiagnosis and missed diagnosis. Clinical studies have demonstrated that COVID-19 has characteristic manifestations in chest CT. According to the CT imaging manifestations of 65 COVID-19 patients, the comparison of the imaging manifestations between the two groups of patients displayed that the latter three manifestations in the moderate-to-severe group were significantly higher than those in the moderate group ($t = 10.92$, $P < 0.05$). It is necessary to note that COVID-19 patients rarely present with pleural effusion or lymphadenopathy (Medical Expert Group of Tongji Hospital Affiliated to Tongji Medical College of Huazhong University of Science Technology, 2020).

For antiviral treatment, the treatment scheme in "the Diagnosis and Treatment Scheme of the Pneumonia Caused by the Novel Coronavirus" established by the National Health Commission of the People's Republic of China has been constantly adjusted. α- interferon, lopinavir/ritonavir, abidor tablets, ribavirin, and chloroquine phosphate, can be trialed. With the ever-changing treatment courses, the joint application of three or more antiviral drugs is not recommended. For the vast majority of the studied cases, we adopted two antiviral drugs. For a small number of young patients, we tried three antiviral drugs with the consent of patients, which had no apparent effect on preventing the moderate patients from developing into severe condition, and the difference between the two groups was not statistically significant ($P > 0.05$). Meanwhile, the time for the virus to turn negative was not shortened, and a proportion of patients even had adverse reactions. So, we do not recommend the use of more than two antiviral drugs, the exact efficacy of which needs further clinical observation.

In our study, the majority of the 24 moderate-to-severe patients developed into moderate condition after active treatment, one patient developed into critical condition, and all these patients were cured and discharged eventually. In conclusion, the risk factors for COVID-19 patients to develop from moderate to severe condition consists of: complicated common underlying diseases, respiratory frequency, lymphocyte count, blood glucose, albumin, urea, inflammatory factor (CRP, IL-6), and imaging manifestations. It is worth noting in clinical work that early detection and treatment is crucial to raise the cure rate and reduce the mortality. However, due to limited number of cases in this study, it may be necessary to conduct a meta-analysis of the relevant parts in further research to make the results more convincing.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the First Affiliated Hospital of Bengbu Medical College (2020ky010).

AUTHOR CONTRIBUTIONS

DL, YY, CL, and YZ conceived and designed the study. DL, JH, and YZ contributed to the literature search. DL, JL, and YZ contributed to data collection. DL, JH, and YY contributed to data analysis. DL and YY contributed to data interpretation. DL, CL, and JH contributed to the figures. YY and DL contributed to writing of the report. All authors contributed to the article and approved the submitted version.

REFERENCES

Castrucci, M. R. (2018). Factors affecting immune responses to the influenza vaccine. *Hum. Vaccines Immunother.* 14, 637–646. doi: 10.1080/21645515.2017.1338547

Chan, J. F., Yuan, S., Kok, K. H., To, K. K., Chu, H., Yang, J., et al. (2020). A familial cluster of pneumonia associated with the 2019 novel coronavirus indicating person-to-person transmission: a study of a family cluster. *Lancet* 395, 514–523. doi: 10.1016/S0140-6736(20)30154-9

Chen, L., Liu, H. G., Liu, W., Liu, K., Shang, J., Deng, Y., et al. (2020). Analysis of clinical features of 29 patients with 2019 novel coronavirus pneumonia. *Chin. J. Tuberc. Respir. Dis.* doi: 10.3760/cma.j.issn.1001-0939.2020.0005. [Epub ahead of print].

Chen, Y., Liu, Q., and Guo, D. (2020). Emerging coronaviruses: genome structure, replication, and pathogenesis. *J. Med. Virol.* 92, 418–423. doi: 10.1002/jmv.25681

Guo, L., Wei, D., Zhang, X., Wu, Y., Li, Q., Zhou, M., et al. (2019). Clinical features predicting mortality risk in patients with viral pneumonia: the MuLBSTA score. *Front. Microbiol.* 10:2752. doi: 10.3389/fmicb.2019.02752

Huang, C., Wang, Y., Li, X., and Ren, L. (2020). Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet* 395, 497–506. doi: 10.1016/S0140-6736(20)30183-5

La Gruta, N. L., Kedzierska, K., Stambas, J., and Doherty, P. C. (2007). A question of self-preservation: immunopathology in influenza virus infection. *Immunol. Cell Biol.* 85, 85–92. doi: 10.1038/sj.icb.7100026

Medical Expert Group of Tongji Hospital Affiliated to Tongji Medical College of Huazhong University of Science and Technology (2020). Quick Guide to Novel Coronavirus Infection in Pneumonia, 3rd Edn (EB/OL). Available online at: https://www.tjh.com.cn/html/2020/0128/28713.shtml#title (accessed January 28, 2020).

Novel coronavirus pneumonia diagnosis and treatment plan of People's Republic of China national health and Health Committee (trial version sixth) [EB/OL]. Available online at: http://www.nhc.gov.cn/yzygj/s7653p/202002/8334a8326dd94d329df351d7da8aefc2/files/b218cfeb1bc54639af227f922bf6b817.pdf

Ren, L. L., Wang, Y. M., Wu, Z. Q., Xiang, Z. C., Guo, L., Xu, T., et al. (2020). Identification of a novel coronavirus causing severe pneumonia in human: a descriptive study. *Chin. Med. J.* 133, 1015–1024. doi: 10.1097/CM9.0000000000000722

Wang, D., Hu, B., Hu, C., Zhu, F., Liu, X., Zhang, J., et al. (2020). Clinical characteristics of 138 hospitalized patients with 2019 novel coronavirus-infected pneumonia in Wuhan, China. *JAMA* 323, 1061–1069. doi: 10.1001/jama.2020.1585

Yang, P. H., Ding, Y. B., Xu, Z., Pu, R., Li, P., Yan, J., et al. (2020). Epidemiological and clinical features of COVID-19 patients with and without pneumonia in Beijing, China. *Medrxiv [preprint]*. doi: 10.1101/2020.02.28.20028068

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Public Health

CASE REPORT
published: 15 September 2020
doi: 10.3389/fpubh.2020.553730

Epidemiological Chronicle of the First Recovered Coronavirus Disease Patient From Panama: Evidence of Early Cluster Transmission in a High School of Panama City

Augusto Hernandez[1†], Paul Muñoz[1†], Jose C. Rojas[2†], Gilberto A. Eskildsen[3,4], Julio Sandoval[4], K. S. Rao[3], Rolando A. Gittens[3] and Jose R. Loaiza[3,5,6]*

[1] Unidad de Cuidados Intensivos, Complejo Hospitalario Doctor Arnulfo Arias Madrid, Caja de Seguro Social (CSS), Panama City, Panama, [2] Colegio Monseñor Francisco Beckmann, Panama City, Panama, [3] Centro de Biodiversidad y Descubrimiento de Drogas, Instituto de Investigaciones Científicas y Servicios de Alta Tecnología, Panama City, Panama, [4] Unidad de Cuidados Intensivos, Ministerio de Salud de Panamá (MINSA), Panama City, Panama, [5] Smithsonian Tropical Research Institute, Panama City, Panama, [6] Programa Centroamericano de Maestría en Entomología, Universidad de Panamá, Panama City, Panama

The first patient infected by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in Panama was reported on March 9, 2020. Here, we describe the first case of recovery from coronavirus disease 2019 (COVID-19) in the country. The patient was a 49-year-old male high school teacher, who did not show any primary symptoms of COVID-19 described by health authorities as the signs for medical attention. Nonetheless, he became severely ill over the course of 2 weeks and almost lost the battle against COVID-19. The identification of the first cluster of SARS-CoV-2 community transmission in the secondary school where the patient of this case report taught, led to the closure of the school and, a day after, the shutdown of the national education system, which may have prevented the spread and slowed the transmission rate of COVID-19 during the early stages of invasion. This case report highlights the need to increase awareness among healthcare professionals in Latin America to consider symptoms such as anosmia and dysgeusia as the sentinel signs of COVID-19 infection in order to prevent deaths, especially in high-risk patients.

Keywords: epidemiological investigation, SARS-CoV-2, COVID-19, anosmia, dysgeusia, Panama City

OPEN ACCESS

Edited by:
Zisis Kozlakidis, International Agency for Research on Cancer (IARC), France

Reviewed by:
Siddharth Sridhar, The University of Hong Kong, Hong Kong
Thenral Mangadu, The University of Texas at El Paso, United States
Istvan Boldogh, University of Texas, United States
Taryn Clark, SUNY Downstate Medical Center, United States

***Correspondence:**
Jose R. Loaiza
jloaiza@indicasat.org.pa

[†]These authors have contributed equally to this work

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Public Health

Received: *20 April 2020*
Accepted: *14 August 2020*
Published: *15 September 2020*

Citation:
Hernandez A, Muñoz P, Rojas JC, Eskildsen GA, Sandoval J, Rao KS, Gittens RA and Loaiza JR (2020) Epidemiological Chronicle of the First Recovered Coronavirus Disease Patient From Panama: Evidence of Early Cluster Transmission in a High School of Panama City. Front. Public Health 8:553730. doi: 10.3389/fpubh.2020.553730

INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the etiological agent of the coronavirus disease (COVID-19) pandemic that has affected more than 100 countries, with more than 19.3 million confirmed cases (1). High fever (38°C), dry cough, and shortness of breath are the most common symptoms resulting in patients requiring oxygen treatment, and some needing immediate access to the intensive care unit (ICU) due to respiratory distress with ≥50% probability of death (2). Globally, a lethality rate ranging from 2.1% (South Korea) to 14.3% (Italy) has been reported, depending on case surveillance strategy and number of tests (per million people) across countries affected by COVID-19 (1–3). The infection severely affects people >60 years of age, while children and young adults are often oligosymptomatic. Nonetheless, the infection could be

dangerous in younger individuals with underlying diseases (2, 3). With no specific antiviral drug therapy or an effective vaccine against SARS-CoV-2 in the near future, patients under critical conditions are treated with the standard supportive care practices for acute respiratory distress syndrome (4). At the same time, public health outbreak response measures are based on enforcing isolation, quarantine, and social distancing to mitigate the spread of the disease, and reduce the number of people requiring hospitalization (3–5).

Panama City is the second most populous urban center in Central America and a hub of international trade and tourism. It has a metropolitan population of 1.6 million people, and ∼2.5 million visitors arriving from abroad annually (6). The Ministry of Health in Panama (MINSA, for its initials in Spanish), with the support of the Pan-American Health Organization, had established a strong containment strategy that covered all ports of entry into the country since March 16, 2020. Extra stringent actions of community containment such as closing schools and universities, private sector companies, and government offices were also implemented by March 12, 2020, including limiting large gatherings of people in commercial centers, sport arenas, and other public spaces (7). The main goal of these actions was to reduce the transmission rate of the virus for 14–20 days after a cluster of activity had been identified, and protect the overall healthcare system capacity.

Currently, attempts to prevent the spread of the virus are no longer focused only on the close contacts of confirmed cases within familial households or work-related spaces. Strict social distancing measures are being implemented to help decrease the spread of the virus, but the curve has not flattened yet. As of August 7, 2020, SARS-CoV-2 has infected 71,418 people in the country, and 1,574 have died (1, 8). Although it is believed that the initial infections in Panama originated from travelers who entered the country from Europe and the US (9, 10), the epidemiological scenario surrounding a potential index case has not yet been established in the country. In this report, we describe the epidemiological chronicle of the first COVID-19 recovery case in Panama. This patient is a high school teacher in Panama City, who initially had no fever or dry cough, but instead presented loss of appetite, anosmia, and dysgeusia along with episodes of mild-to-moderate dyspnea that worsened over 2 weeks.

CASE REPORT

Written and signed informed consent was obtained from the patient to publish this case report. On February 21, 2020, a 49-year-old male with flu-like symptoms such as myalgia, dehydration, fatigue, and chills, but without fever, cough, or respiratory distress visited the hospital to seek medical treatment. He was sent back home with prescriptions by the medical doctor for a probable viral infection. At that point, there were no reported cases of COVID-19 in Panama. Prior to this time, the patient was healthy, without any preexisting medical conditions and had not traveled outside Panama in the last 12 months. As the symptoms continued, including additional ones such as diarrhea, dizziness, and dyspnea, the patient visited a private clinic twice in <6 days, and tested negative for dengue virus (**Figure 1**). A week after symptom onset, his body temperature (37.0°C, 98.6°F) and total white blood cell count (9.2 × 103 cells/μl) were in the normal range, but he would easily get tired after simple activities such as walking up the stairs of the school. During the first days of the academic year in Panama (March 1–3, 2020), he was unable to smell (anosmia) or taste any food (dysgeusia). He had diminished appetite but decided to continue teaching biology to ∼40 teenage students.

On March 3, 2020, he was admitted to the public hospital Caja de Seguro Social (CSS) Complejo Hospitalario Doctor Arnulfo Arias Madrid, along with the 64-year old male director of the same school, who had high fever, severe cough, and persistent respiratory problems. It is important to note that this occurred 6 days before MINSA officially confirmed the first case of COVID-19 in Panama in a Panamanian citizen who had recently returned from Spain on March 8, 2020. On arrival, the high school teacher's symptoms had evolved to dry cough, fever, and shortness of breath (**Figure 1**), and his chest radiograph showed extensive areas of multilobar opacities and bilateral minor pleural effusions, suggesting pneumonia (**Figures 2A–D**) (11). At that point, the complete blood count (CBC) showed leukocytosis (82.1% neutrophils) and hyperglycemia (**Table 1**). These measurements exceeded or were short of the normal range from day 2 until day 18 of hospitalization. In addition, CBC from day 8 to day 21 showed low levels of hemoglobin and hematocrit (**Table 1**). Moreover, on March 3, 2020, he was tested for a panel of respiratory diseases/pathogens by the Film Array polymerase chain reaction (PCR) technique (BioFire; Salt Lake City, USA), including several strains of coronavirus known to infect humans, and other numerous viral and bacterial pathogens. However, all results were categorized as undetected (**Table 1**). Additional laboratory testing was completed on day 2 of hospitalization, including microbiological culture, urinalysis, and serum chemistry. Between day 1 and day 18, the patient presented leukocytosis with neutrophilia, lymphopenia, and hypoalbuminemia (**Table 1**). From day 2–21, there were some alterations in the hepatic function, including higher levels of alanine transferase (ALT), aspartate transferase (AST), and lactate dehydrogenase (LDH) (**Table 1**). The chest radiographs continued to show signs of bilateral alveolar infiltrates until day 20, but signs of improvement were seen from March 12 (**Figures 2E–G**). Despite not knowing the cause of the patient's illness initially, on March 6, 2020, the medical team decided to initiate treatment with cephalosporins and continuous infusion of methylprednisolone (100 mg/24 h) for 4 days (**Figure 1**).

Given the many tropical infectious diseases with similar symptomologies to COVID-19 in Latin America, the medical staff thought the patient's presentation was most likely caused by a local infection. Also, since no confirmed case of COVID-19 had been reported in Panama at the time when the patient was admitted to the hospital, and the World Health Organization (WHO) had not yet declared the pandemic, he was not tested for SARS-CoV-2 using reverse-transcription-PCR (RT-PCR). Instead, the medical staff suspected that he had contracted the Hantavirus (HTV) or Sin Nombre virus of the *Bunyaviridae*

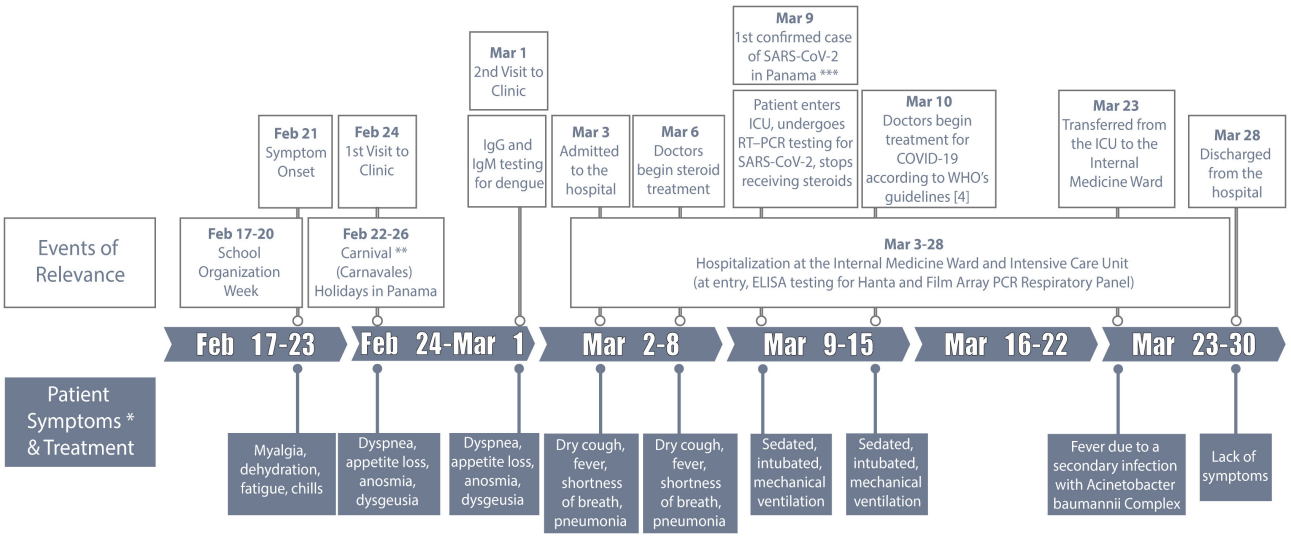


FIGURE 1 | Case history of the first recovered coronavirus disease (COVID-19) patient from Panama. *The patient experienced no fever, sore throat, nasal congestion, running nose or severe coughing since symptom onset until the day of hospitalization. **Carnivals ("Carnavales") are one of the most popular holiday celebrations of the year in Panama. ***The first confirmed case of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in Panama using reverse-transcription polymerase-chain-reaction (RT-PCR) testing was reported on March 9, 2020, and the same day the patient was confirmed positive for SARS-CoV-2 infection. The school where the first cluster of activity was detected in Panama City closed on March 10, 2020, 5 days after the beginning of the academic year. ICU, Intensive care unit; IgG, Immunoglobulin G.

family, an endemic and rarely fatal infection that causes severe pulmonary and renal complications in humans (12). Indeed, he had been in the Azuero peninsula visiting relatives during a family gathering 3 weeks earlier, which is the area with the most number of HTV cases per year recorded in Panama. He could have potentially been exposed to urine secretions from the animal reservoir (e.g., *Oligoryzomys fulvescens*) (12). However, the result for HTV test was negative. After receiving information from MINSA about the first confirmed COVID-19 case in the country, which was after 6 days of being hospitalized and more than 13 days of symptom onset, the patient underwent RT-PCR and tested positive for SARS-CoV-2 RNA. On March 6, 3 days before the SARS-CoV-2 infection was confirmed in the patient, his respiratory pattern worsened together with deterioration of the oxygenation parameters, requiring intubation and invasive mechanical ventilation. He was transferred to the ICU on March 9 (**Figure 1**; **Table 1**), where the medical team suspended treatment with steroids and began treatment with hydroxychloroquine, azithromycin, and lopinavir/ritonavir regimen according to WHO recommendations (4).

An epidemiological investigation began almost immediately to determine all contacts and potentially newly infected people. Approximately 15 people, including family members, students, friends, and colleagues were tested for SARS-CoV-2, none of whom tested positive or became ill with COVID-19. In addition, ∼200 people from the school who had been in close contact with the director and the patient were followed-up clinically, and 10% were tested for SARS-CoV-2. The patient still does not know where he may have acquired the virus, as he had not been in close contact with the director or co-worker during the school organization week (**Figure 1**). Instead, cluster transmission might have been already occurring at the school, as seven more professors tested positive for SARS-CoV-2, after this patient was tested for the virus. Interestingly, none of these people or their relatives had traveled outside Panama in the last 12 months, and none developed severe symptoms or died due to COVID-19. As part of the first officially reported cluster of COVID-19 cases in the country, this case report served as evidence for health authorities to close the patient's school on March 10, 2020 (13), and shutdown the national education system just a day after (14).

Due to recurrent fever and neutrophilia, blood cultures and respiratory samples were obtained from the patient on March 18 (day 16 of hospitalization), showing an *Acinetobacter baumannii* complex infection without organ dysfunction, which was treated successfully (**Figure 1**). On March 19, the patient was extubated, weaned off the invasive mechanical ventilation, and discharged from the ICU after 4 days. He was officially designated as the first Panamanian resident to have recovered from an aggravated case of COVID-19 disease in the country (15). The patient's co-worker and director of the high school died on March 8, 2020, 5 days after being hospitalized. The autopsy revealed a prior infection with SARS-CoV-2 and resultant death due to COVID-19.

DISCUSSION

Panama was one the of the first Latin America countries to enter the list of territories affected by the COVID-19 pandemic. It has the largest testing rate per inhabitant in the region, and

TABLE 1 | Clinical results of the first recovered coronavirus disease (COVID-19) patient from Panama.

Measure	Reference range	Day 1 Mar 3	Day 2 Mar 4	Day 8 Mar 10	Day 10 ICU Mar 12	Day 16 ICU Mar 18	Day 18 ICU Mar 20	Day 21 Mar 23
White cell count (/μl)	3.9–11.5 × 10^3	13.0	15.3	17.5	16.8	25.5	14.7	7.6
% Neutrophil	50–70	82.1	88	86.2	89.9	91.5	81	59.4
Platelet count (/μl)	150–400	281	249	464	515	455	342	410
% Lymphocytes	25–50	8.5	6.2	6.5	6.7	2.7	10.2	22.2
Red blood cell count (per μl)	4.0–6.2 × 10^6	4.51	4.0	3.57	3.37	3.32	3.74	3.8
Hemoglobin (g/dl)	12.5–18	12.7	12.5	11.3	10.5	9.9	11.2	11.5
Hematocrit (%)	36–50	41.9	37.7	35	32.2	32.3	35.4	35.8
Glucose (mg/dl)	70–105	126	151	119	115	178	77	80
Blood urea nitrogen (mg/dl)	6–20	11	12	18	21	35	22	25
Creatinine (mg/dl)	0.7–1.2	1.07	0.85	0.80	0.65	0.59	0.60	0.60
Albumin (g/dl)	3.4–4.8	-	2.8	2.7	2.6	3.1	2.9	3.4
Sodium (mEq/liter)	136–145	137	136	146	145	145	138	140
Potassium (mEq/liter)	3.5–5.1	4.3	3.9	4.5	4.0	4.4	3.1	4.7
Chloride (mEq/L)	98–107	97	100	103	103	106	100	104
Alanine transferase (U/L)	10–50	-	146	150	282	530	241	133
Aspartate transferase (U/L)	10–38	-	178	107	252	146	50	48
Lactate dehydrogenase (U/L)	120–230	-	372	424	605	414	335	356
Creatine phosphokinase (U/L)	38–174	-	325	371	907	-	127	76
C-Reactive protein (U/L)	0–3	-	-	-	-	16.7	96	-
Culture (Endotracheal Secretion)	n/a	-	-	-	-	***	-	-
*Film Array PCR Respiratory Panel	n/a	**	-	-	-	-	-	-

Color code: uncolored boxes, values within the reference range; green boxes, values below the reference range; red boxes, values above the reference range; gray boxes, values not measured.

ICU, Intensive care unit.

*A nasopharyngeal swab specimen was tested for a respiratory panel of human pathogens and illness by *Film Array polymerase chain reaction (BioFire; Salt Lake City, UT, USA), including various viruses (Coronavirus 229E, Coronavirus HKU1, Coronavirus OC43, Coronavirus NL63, Adenovirus, Human Metapneumovirus, Human Rhinovirus/Enterovirus, Influenza A, Influenza A/H1, Flu-A-H1 2009, Flu-A-H1 PAN, Flu-A-H3, Flu-A-PAN-1, Flu-A-PAN-2, Influenza B, Parainfluenza virus 1, Parainfluenza virus 2, Parainfluenza virus 3, Parainfluenza virus 4, and VRS) and bacteria (Bordetella pertussis, Chlamydophila pneumoniae, and Mycoplasma pneumonia).*

***Not detected; ***Acinetobacter baumannii complex associated health care infection.*

consequently the highest incidence of COVID-19 with 71,418 confirmed cases and 1,574 deceased by day 151 since the initial invasion (8). This makes it an ideal location to outline the potential epidemiological scenarios that might be present in the early stages of SARS-CoV-2 invasion in other countries of this region. Here, we described the epidemiologic chronicle of the first COVID-19 recovery case from Panama. The patient was a 49-year-old male high school teacher, who did not show any of the primary COVID-19 symptoms initially (i.e., fever, dry cough, and respiratory distress) as described by health authorities for seeking medical attention (16, 17). Nonetheless, he became severely ill over the course of 2 weeks and almost lost the battle against COVID-19.

Despite having severe pneumonia and being critically ill for 17 days, the medical staff claimed that he recovered quickly from COVID-19 because he did not have any underlying conditions such as hypertension, diabetes, and cardiovascular or renal diseases (17). From a clinical stand point, it is noteworthy to mention that the 4-days course of continuous infusion of steroids seems to have had a positive impact on the clinical condition of the patient, evidenced by the significant radiological improvement from March 9 to 20 (**Figures 2D–G**). However, steroid treatment in COVID-19 patients has been controversial, with inconsistent clinical outcomes (4, 16, 17). Similar to other COVID-19 case studies, the patient showed signs of lymphopenia and 16% increased levels of aspartate amino transferase (17–19). Further laboratory testing confirmed lymphopenia and elevated values of AST, ALT, C-reactive (CRP), and LDH levels. Nevertheless, leukocytosis was detected in the patient upon arrival at the hospital and remained until day 18 of hospitalization. This finding differed from previous publications where up to 31% of COVID-19 patients showed consistent signs of leukopenia (18–20). On day 18, his leukocyte count was 25.5 × 10^3 cells/μl, due to an infection with the gram-negative bacterium *Acinetobacter baumannii* complex, which is one of the most frequent opportunistic pathogens causing hospital-acquired infections worldwide.

OUTBREAK INVESTIGATION

Based on the case history, it appears that the patient was infected with SARS-CoV-2 before the "Carnivals" (*Carnavales* in Spanish), as he began feeling ill around February 21 and sought medical attention for the first time on February 24.

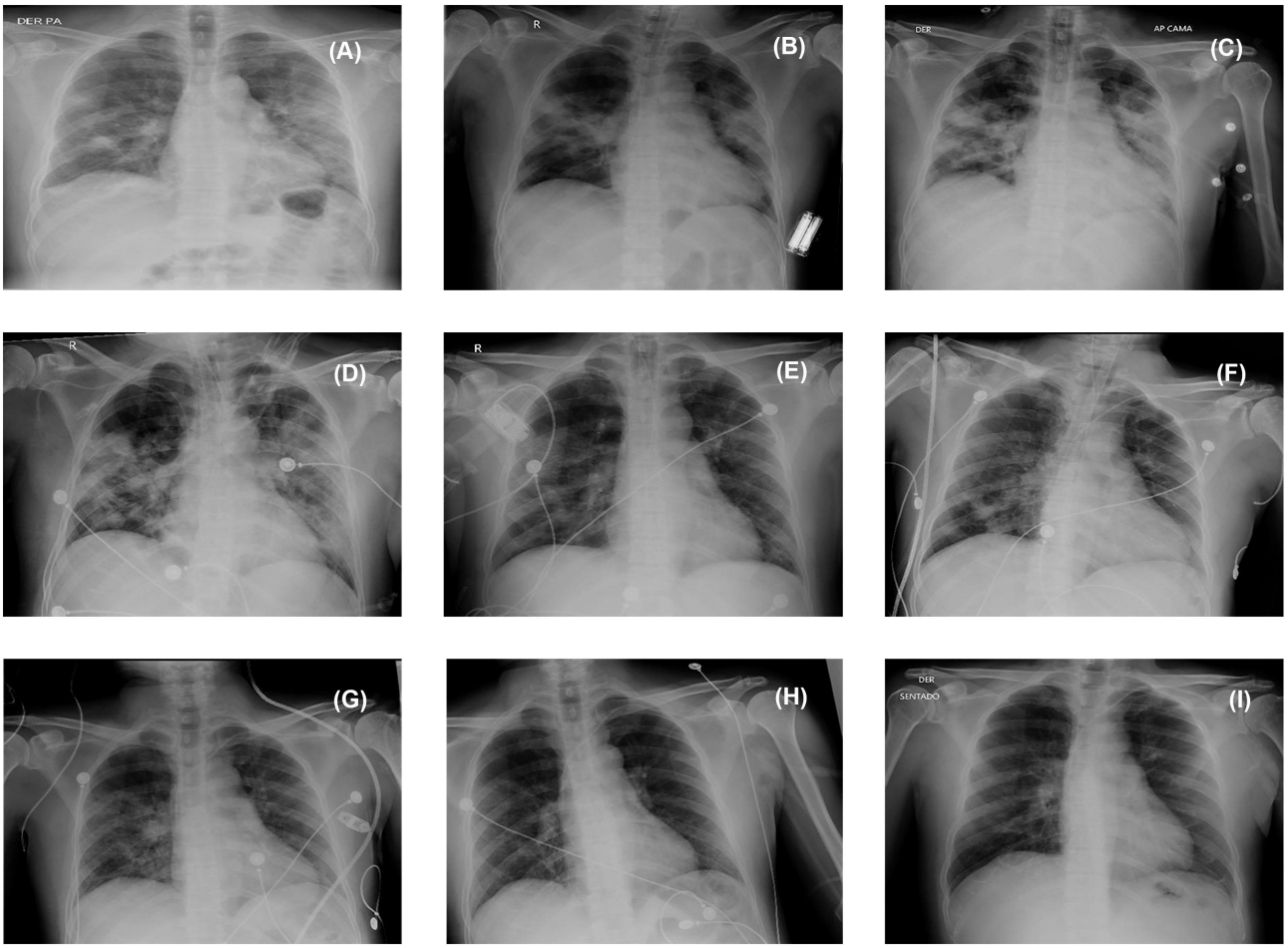


FIGURE 2 | Chest radiographs. The radiographs show bilateral ground-glass opacities **(A)** on admission to the hospital on March 3, 2020; **(B)** during intubation on March 5; **(C)** during begin treatment with steroids on March 6; **(D)** on entering the intensive care unit on March 9; **(E)** on March 12; **(F)** on March 17; and **(G)** on March 20. No alveolar infiltrates observed on the radiographs **(H)** on March 23 and **(I)** during discharge from intensive care unit on March 24, 2020.

The *Carnavales* started on February 22 and lasted for 4 days until February 26, 2020 (**Figure 1**) (21). *Carnavales* is one of the most popular holiday celebrations of the year in Panama, where thousands of locals and foreigners travel throughout the country to celebrate in social spaces or visit their family homes in the countryside. Whether or not the arrival and spread of SARS-CoV-2 in Panama can be associated with *Carnavales* remains to be answered, but such movements and gatherings could have facilitated numerous close contacts with potentially infected people over a short period of time and across long distances (17). The initial transmission of SARS-CoV-2 in Panama might have occurred during the preparation week (February 17–20), when 206 professors and 60 administrative workers shared the school with the first cluster of COVID-19 cases in the country, including our case in point. Some of these workers might have traveled overseas during late January and early February of 2020 for their annual vacation. Closing the school on March 10, 2020 a week after the beginning of the academic year, and immediately after becoming the first cluster of COVID-19 cases in the country, helped to reduce exposure to the virus and stopped further transmission among 4,200 teenaged students. This is corroborated by the lack of symptoms or deaths among the students from that school until now. To the best of our knowledge, there has not been any report published in the literature about the effectiveness of school closure in stopping the spread of SARS-CoV-2 infection to other geographic regions. In fact, this is currently a subject of much debate, and there is not much evidence against or in favor of this containment strategy. Our case report stands as an early circumstantial evidence that school closure could in fact be beneficial to inhibit the spread of SARS-CoV-2 to a larger portion of the academic community.

Panamanian health authorities must be on the lookout for early symptoms, including appetite loss, anosmia, and dysgeusia, and use them as sentinel signs of COVID-19 disease in order to treat people in a timely and effective manner (22–24). This is especially important to avoid further fatalities in individuals from the high-risk group, including those over 65 years of age, smokers, and/or those with co-morbidities (i.e., hypertension, diabetes, and cardiovascular or renal diseases). These symptoms can also help to differentiate between COVID-19 disease and

other sign-related illnesses such as dengue, influenza, or HTV, especially now that some of these diseases are likely to emerge with the beginning of the rainy season in Panama. Dengue virus and HTV are just two examples of many other tropical diseases that are not observed in the northern hemisphere (e.g., Europe and the US), which were initially suspected as the cause of the disease in this patient. Therefore, not considering the diversity of zoonotic tropical pathogens that can cause misdiagnosis of COVID-19 could be a problem in the future as this emergent disease is likely to become the next neglected infection in the poor communities of tropical countries. Our report implies that SARS-CoV-2 must be included in the panel of respiratory infectious diseases to routinely test for potential COVID-19 cases, especially in the post-pandemic era.

This report suggests that patients who do not experience aggressive coughing and/or high fever in the early stages of infection might not spread the virus actively. Therefore, the use of face masks in public spaces along with actions of self-quarantine and social distancing are highly recommended to disrupt further transmission. Primary and secondary schools as well as universities must remain closed in Panama until sufficient herd immunity has been acquired in the susceptible population, and COVID-19 asymptomatic carriers no longer seem to be a threat to the local healthcare system. Homeschooling and online teaching could ultimately prevent waves of SARS-CoV-2 infections in the future due to reduced transmission rates.

LIMITATIONS

We know that this is in fact the first COVID-19 patient who recovered from an episode of aggravated respiratory illness; therefore, he might have been closely associated with the first infected person who came to the school. A possible contact between our patient and a pre-symptomatic COVID-19 person cannot be ruled out, especially because this phenomenon has been reported in the early stages of an outbreak (25). However, due to the lack of epidemiological information from the other seven COVID-19 positive patients of the school, we cannot corroborate this possibility, nor can we discuss additional transmission scenarios among all these patients. After closure of the school by health authorities on March 10, 2020, 12 more people, mostly middle-aged adults, reported respiratory problems. However, confirmatory RT-PCR testing was not performed for these patients; hence, it is unknown if they were infected with SARS-CoV-2.

CONCLUSION

The patient described in this case report was among the first patients admitted to the ICU in a public hospital in Latin America. Furthermore, he was among the first survivors, notwithstanding the fact that the doctors did not treat him with therapeutic practices and medication specifically targeting a SARS-CoV-2 infection. Our patient was treated for atypical pneumonia after 4 days of being hospitalized and more than 13 days of symptom onset. Surviving an infection with a new and deadly pathogen is a remarkable and fascinating clinical outcome considering the limited knowledge and preparation that the Panamanian medical staff had at the beginning of this invasion. The closure of a secondary school in Panama City due to the identification of the first cluster of SARS-CoV-2 activity, triggered the immediate shutdown of the education system in the entire country, which may have prevented the spread and slowed the transmission rate of COVID-19 during the early stages of invasion.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

AH, PM, JR, and JL wrote the first draft of the manuscript with contributions from GE, JS, KR, and RG. GE, AH, PM, JS, and KR evaluated and analyzed the clinical history and laboratory testing of the patient. AH and PM treated the patient at the hospital. All authors read and approved the final manuscript.

FUNDING

The National System of Investigation of Panama (SNI) at SENACYT supports research activities by JL (05-2016 & 157-2017), KR (156-2017 & 062-2019, and RG (91-2015 & 146-2017).

ACKNOWLEDGMENTS

We are grateful to the epidemiological personnel at the Panamanian Ministry of Health and the Authority of Government Innovation for sharing the metadata about the geographic localities where COVID-19 cases had been reported in Panama by July 26, 2020. We are thankful to Melanie R. Loaiza Santos for compiling and preparing the panels of **Figure 2**.

REFERENCES

1. World Health Organization. *Novel Coronavirus (COVID-19) Situation*. World Health Organization (2020). Available online at: https://experience.arcgis.com/experience/685d0ace521648f8a5beeeee1b9125cd (accessed March 9, 2020).
2. Wu Z, McGoogan JM. Characteristics of and important lessons from the coronavirus disease 2019 (COVID-19) outbreak in china: summary of a report of 72314 cases from the chinese center for disease control and prevention. *JAMA*. (2020) 323:1239–42. doi: 10.1001/jama.2020.2648
3. McCloskey B, Heymann DL. SARS to novel coronavirus - old lessons and new lessons. *Epidemiol Infect*. (2020) 148:e22. doi: 10.1017/S0950268820000254

4. World Health Organization. *Clinical Care for Severe Acute Respiratory Infection: Toolkit: COVID-19 Adaptation*. Geneva: World Health Organization (2020). Report No.: Contract No.: WHO/2019-nCoV/SARI_toolkit/2020.1.
5. Wilder-Smith A, Freedman DO. Isolation, quarantine, social distancing and community containment: pivotal role for old-style public health measures in the novel coronavirus (2019-nCoV) outbreak. *J Travel Med*. (2020) 27:taaa020. doi: 10.1093/jtm/taaa020
6. Goñi E, López HJ, Servén L. Fiscal redistribution and income inequality in latin America. *World Dev*. (2011) 39:1558–69. doi: 10.1016/j.worlddev.2011.04.025
7. MINSA. *Plan Operativo Nacional del Sistema de Salud para la Prevención y Control del nuevo Coronavirus (2019-vCoV)*. Panama: MINSA (2020).
8. MINSA, ICGES. *Seguimiento de Casos COVID-19 en Panamá*. Panama: MINSA (2020). Available online at: https://geosocial.maps.arcgis.com/apps/opsdashboard/index.html#/2c6e932c690d467b85375af52b614472 (accessed MAy 11, 2020).
9. Franco D, Gonzalez C, Abrego LE, Carrera JP, Diaz Y, Caisedo Y, et al. Early transmission dynamics, spread, and genomic characterization of SARS-CoV-2 in Panama. *medRxiv [Preprint]*. (2020) 2020.07.31.20160929. doi: 10.1101/2020.07.31.20160929
10. Samaniego C. A. *Gorgas: Cepas del virus llegaron todas a Panamá entre febrero y marzo Panama*. (2020) Available online at: https://www.prensa.com/impresa/panorama/gorgas-cepas-del-virus-llegaron-todas-a-panama-entre-febrero-y-marzo/ (updated May 3; accessed June 2, 2020).
11. Zu ZY, Jiang MD, Xu PP, Chen W, Ni QQ, Lu GM, et al. Coronavirus disease 2019 (COVID-19): a perspective from China. *Radiology*. (2020) 296:E15–E25. doi: 10.1148/radiol.2020200490
12. Armien B, Pascale JM, Munoz C, Marinas J, Nunez H, Herrera M, et al. Hantavirus fever without pulmonary syndrome in Panama. *Am J Trop Med Hyg*. (2013) 89:489–94. doi: 10.4269/ajtmh.12-0334
13. Morales Gil E. El caso del colegio Beckmann, ¿transmisión comunitaria? *Panama: La Prensa*. (2020). Available online at: https://www.prensa.com/impresa/panorama/el-caso-del-colegio-beckman-transmision-comunitaria/ (updated Mar 13; accessed March 20, 2020).
14. Molina UC. *El Meduca suspende las clases en todo el país hasta el 20 de marzo; Panamá centro, norte y San Miguelito, hasta el 7 de abril*. Panama: La Prensa (2020). Available online at: https://www.prensa.com/sociedad/meduca-ordena-suspension-de-clases-en-todo-el-pais-hata-el-20-de-marzo/ (updated Mar 11; accessed March 12, 2020).
15. Redacción. *Coronavirus en Panamá: Profesor del Colegio Francisco Beckmann sale de la Unidad de Cuidados Intensivos*. Panama: Panamá América (2020). Available online at: https://www.panamaamerica.com.pa/sociedad/coronavirus-en-panama-profesor-del-colegio-francisco-beckmann-sale-de-la-unidad-de-cuidados (updated Mar 25; accessed May 15, 2020).
16. Zhang J, Zhou L, Yang Y, Peng W, Wang W, Chen X. Therapeutic and triage strategies for 2019 novel coronavirus disease in fever clinics. *Lancet Respir Med*. (2020) 8:e11–e2. doi: 10.1016/S2213-2600(20)30071-0
17. World Health Organization. *Coronavirus disease 2019 (COVID-19) Situation Report – 53*. Geneva: WHO (2020).
18. Kraemer MUG, Yang C-H, Gutierrez B, Wu C-H, Klein B, Pigott DM, et al. The effect of human mobility and control measures on the COVID-19 epidemic in China. *Science*. (2020). 368:493–7. doi: 10.1126/science.abb4218
19. Grasselli G, Zangrillo A, Zanella A, Antonelli M, Cabrini L, Castelli A, et al. Baseline characteristics and outcomes of 1591 patients infected with SARS-CoV-2 admitted to ICUs of the Lombardy Region, Italy. *JAMA*. (2020) 323:1574–81. doi: 10.1001/jama.2020.5394
20. Xu XW, Wu XX, Jiang XG, Xu KJ, Ying LJ, Ma CL, et al. Clinical findings in a group of patients infected with the 2019 novel coronavirus (SARS-Cov-2) outside of Wuhan, China: retrospective case series. *BMJ*. (2020) 368:m606. doi: 10.1136/bmj.m606
21. Morales Gil E. *El coronavirus llegó a Panamá antes de las fiestas de carnaval*. Panama: La Prensa (2020). Available online at: https://www.prensa.com/impresa/panorama/el-coronavirus-llego-a-panama-antes-de-las-fiestas-de-carnaval/ (updated March 14; accessed March 20, 2020).
22. Gostic K, Gomez AC, Mummah RO, Kucharski AJ, Lloyd-Smith JO. Estimated effectiveness of symptom and risk screening to prevent the spread of COVID-19. *Elife*. (2020) 9:e55570. doi: 10.7554/eLife.55570
23. Lorenzo Villalba N, Maouche Y, Alonso Ortiz MB, Cordoba Sosa Z, Chahbazian JB, Syrovatkova A, et al. Anosmia and dysgeusia in the absence of other respiratory diseases: should COVID-19 infection be considered? *Eur J Case Rep Intern Med*. (2020) 7:001641. doi: 10.12890/2020_001641
24. Sivakorn C, Luvira V, Muangnoicharoen S, Piroonamornpun P, Ouppapong T, Mungaomklang A, et al. Case report: walking pneumonia in novel coronavirus disease (COVID-19): mild symptoms with marked abnormalities on chest imaging. *Am J Trop Med Hyg*. (2020) 102:940–2. doi: 10.4269/ajtmh.20-0203
25. Tong ZD, Tang A, Li KF, Li P, Wang HL, Yi JP, et al. Potential presymptomatic transmission of SARS-CoV-2, Zhejiang Province, China, 2020. *Emerg Infect Dis*. (2020) 26:1052–4. doi: 10.3201/eid2605.200198

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Public Health

PERSPECTIVE
published: 15 September 2020
doi: 10.3389/fpubh.2020.579559

Early COVID-19 Interventions Failed to Replicate 1918 St. Louis vs. Philadelphia Outcomes in the United States

Aliea M. Jalali[1,2], *Brent M. Peterson*[1] *and Thushara Galbadage*[1]*

[1] Department of Kinesiology and Health Science, Biola University, La Mirada, CA, United States, [2] College of Nursing and Health Care Professions, Grand Canyon University, Phoenix, AZ, United States

The Coronavirus disease 2019 (COVID-19) pandemic has elicited an abrupt pause in the United States in multiple sectors of commerce and social activity. As the US faces this health crisis, the magnitude and rigor of their initial public health response was unprecedented. As a response, the entire nation shutdown at the state-level for the duration of a ∼1–3 months. These public health interventions, however, were not arbitrarily decided, but rather, implemented as a result of evidence-based practices. These practices were a result of lessons learned during the 1918 influenza pandemic and the city-level non-pharmaceutical interventions (NPIs) taken across the US. During the 1918 pandemic, two model cities, St. Louis, MO, and Philadelphia, PA, carried out two different approaches to address the spreading disease, which resulted in two distinctly different outcomes. Our group has evaluated the state-level public health response adopted by states across the US, with a focus on New York, California, Florida, and Texas, and compared the effectiveness of reducing the spread of COVID-19. Our assessments show that while the states mentioned above benefited from the implementations of early preventative measures, they inadequately replicated the desired outcomes observed in St. Louis during the 1918 crisis. Our study indicates that there are other factors, including health disparities that may influence the effectiveness of public health interventions applied. Identifying more specific health determinants may help implement targeted interventions aimed at preventing the spread of COVID-19 and improving health equity.

Keywords: evidence-based practice, health disparities, coronavirus, spread, intervention, prevention, outcomes, influenza virus

OPEN ACCESS

Edited by:
Zisis Kozlakidis, International Agency For Research On Cancer (IARC), France

Reviewed by:
Enahoro Iboi, Spelman College, United States
Venu Madhav Konala, Ashland Bellefonte Cancer Center (ABCC), United States

***Correspondence:**
Thushara Galbadage
don.galbadage@biola.edu

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Public Health

Received: *02 July 2020*
Accepted: *12 August 2020*
Published: *15 September 2020*

Citation:
Jalali AM, Peterson BM and Galbadage T (2020) Early COVID-19 Interventions Failed to Replicate 1918 St. Louis vs. Philadelphia Outcomes in the United States. Front. Public Health 8:579559. doi: 10.3389/fpubh.2020.579559

INTRODUCTION

As the first wave of Coronavirus Disease 2019 (COVID-19) pandemic began to sweep through the United States (US) in March 2020, multiple public health measures were enforced across the nation in an unprecedented manner. However, by the end of June 2020, the US remained one of the largest COVID-19 epicenters, globally, with more than 2.5 million confirmed cases and the number of new daily cases reaching highs in certain states and the US (1). Now, faced with the renewed threat of experiencing prolonged second wave, many states are reintroducing partial shutdown measures,

which are examples of non-pharmaceutical interventions (NPIs). During the first wave of this pandemic, the US strictly implemented multiple NPIs to help mitigate the spread of the disease and reduce the number of COVID-19-related deaths. Herein we discuss the successes and failures of the implemented evidence-based public health practices amid a nationwide public health crisis that abruptly brought the nation and its economy to a screeching halt.

As of February 2020, while China, Italy, and Spain experienced the turmoil of being the epicenters for the COVID-19 pandemic, the US had only about 50 confirmed cases, and the national populace was nearly unaffected. No one could have anticipated how life was about to change in the ensuing months. In March 2020, different states started to sound the alarms and place their respective constituencies under states of emergency (2–4). After that, increasingly rigorous preventative measures that affected the function and dynamics of societal interaction were implemented. These interventions, aimed at facilitating social distancing and preventing the spread of COVID-19, can be categorized into four broad measures (5, 6). These are (1) screening and testing, (2) prevention of mass gatherings, (3) stay at home orders, and (4) the use of face masks. In the US, 44 states of the 50 states implemented statewide stay at home orders at the early stages of the COVID-19 pandemic, paralleling other measures listed above (**Figure 1**, **Supplemental Table 1**). The mean duration of stay at home orders for all US states was 49.5 days (SD $\pm$ 16.5) (median 50 days, range 25–81 days).

While seemingly sudden and societally intrusive, historical precedent and evidence-based practices have guided these measures. For example, a century ago, the world experienced a devastating toll on lives caused by the 1918 influenza pandemic. In response to this pandemic, health officials implemented a broad range of NPIs according to the then available understanding of disease transmission (8–10). Furthermore, studies comparing public health measures implemented by several cities across the United States and other nations such as England further illustrated how these measures helped reduce the spread of the 1918 influenza pandemic and decrease mortality rates (11–14).

Studies on the 1918 influenza pandemic have focused on contrasting NPIs implemented by two US cities, St. Louis, MO, and Philadelphia, PA. St. Louis imposed strict preventative interventions early on, while Philadelphia minimally applied restrictions at a much later date. Accordingly, St. Louis had a milder outbreak, whereas Philadelphia experienced significantly higher mortality rates (14). These outcomes observed in the 1918 influenza pandemic helped guide the widely-adopted rigorous public health measures against COVID-19. Hatchett et al. (14) also identified four critical factors that helped determine the success of the control of the pandemic dissemination. These factors were (1) implementation of early and rapid interventions, (2) duration of the responses, (3) multiple concurrent interventions, and (4) the intensity of the interventions implemented.

Other studies supported these conclusions while emphasizing the effectiveness of early interventions, but also noted that stringent preventative measures could leave many more susceptible individuals once these NPIs are relaxed (12, 15). During the 1918 pandemic, most of the US cities maintained preventative measures for about 2–8 weeks (14). However, cities that relaxed NPIs earlier experienced increased case numbers resulting in second wave resurgences. An inverse relationship between the intensity of the first and second waves of the pandemic was also observed. These observations were partly due to the smaller proportion of susceptible populations present in cities after a strong first wave of the disease (12, 14).

Here we compare and contrast public health interventions implemented in the US during the first wave of the COVID-19 pandemic, focusing on four states: New York, Florida, Texas, and California. These states included most of the populous US counties and were affected sharply by the early stages of the COVID-19 pandemic. In addition, we studied the case rates of COVID-19 before, during, and after these measures were implemented, and then compared it to the outcomes of St. Louis, and Philadelphia, during the 1918 influenza pandemic (**Figure 2**). While variation in the timing and the intensity of the public health measures applied was observed, all four states implemented very similar interventions. Our comparisons show that the early evidence-based interventions implemented by the US were not adequately able to replicate the desired outcomes of St. Louis vs. Philadelphia and curtail the COVID-19 pandemic.

PUBLIC HEALTH RESPONSE TO COVID-19

As mentioned earlier, responses to earlier pandemics in the US included school closures, restaurant restrictions, emergency declarations, gathering restrictions, stay at home orders, and non-essential business closures (16). The COVID-19-related responses have been mainly relegated to state-level decision making and based on necessity and intensity within each state. To characterize the state-level COVID-19 interventions, we compared and contrasted the broad measured implemented by the states of California, Florida, New York, and Texas.

Screening and Testing

Targeted screening for COVID-19 began in California and New York with Los Angeles (LAX), San Francisco (SFO), and New York (JFK) airports for travelers coming from Wuhan, China, starting on January 17th (17). The first reported case in the US occurred on January 26th in California. New York, Florida, and Texas all had initial cases within the 1st week of March (**Figure 1C**). Early in the pandemic, testing was limited, and priority was given to high-risk individuals, including symptomatic patients, healthcare workers, first responders, essential workers, and individuals in contact with other high-risk individuals. As more tests were readily available, fewer restrictions were placed on who was able to get tested [Florida Department of (18–21)]. In addition to walk-up and drive-through sites, mobile testing sites were also deployed in Florida and New York to increase the number of tests administered (22, 23). Each state also implemented contact tracing to identify potentially exposed individuals (24).

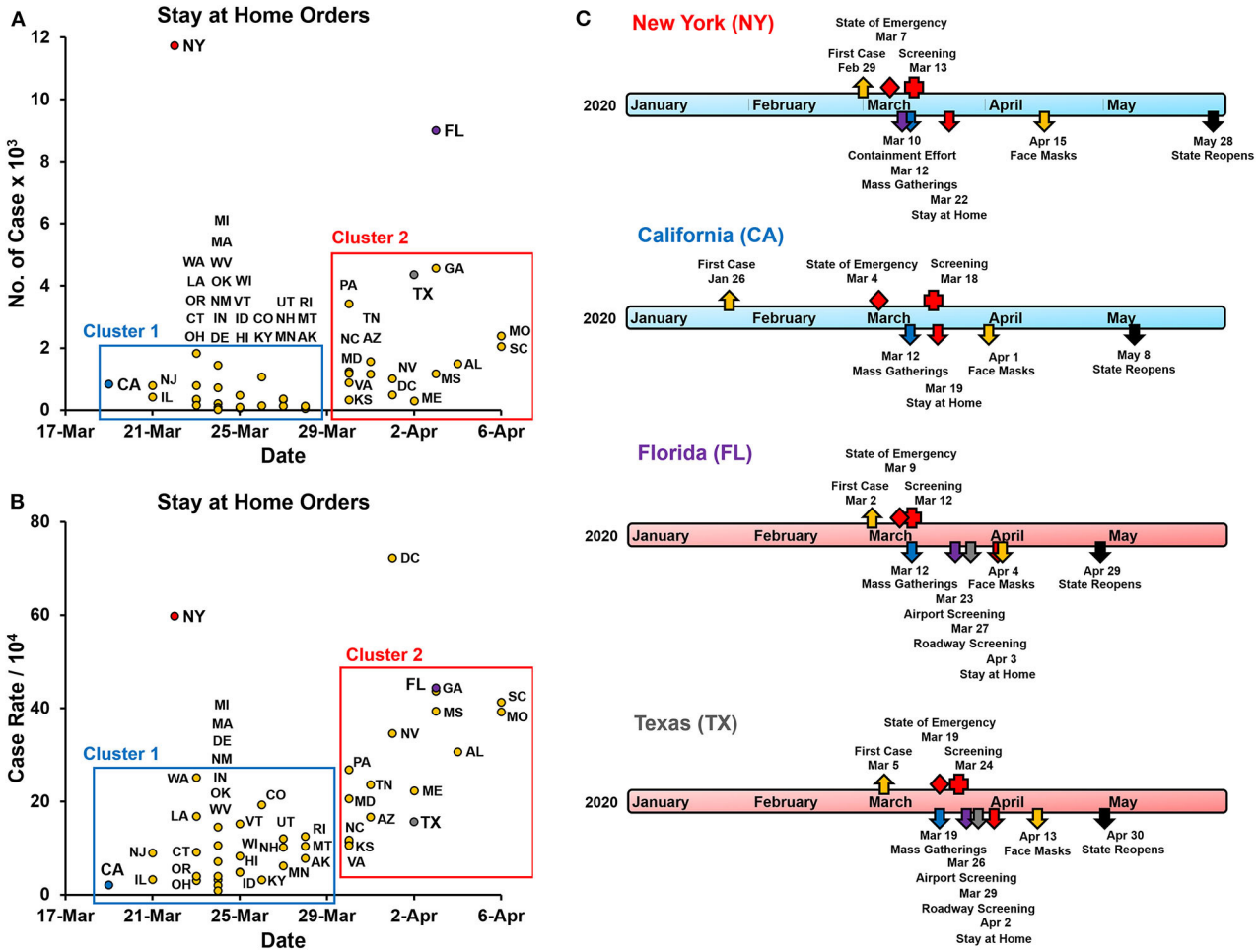


FIGURE 1 | State and county-level public health interventions to contain the spread of COVID-19. **(A)** The number of lab-confirmed COVID-19 cases at the start of the stay at home orders implemented by each state (7). Arkansas, Iowa, Nebraska, North Dakota, South Dakota, and Wyoming did not issue statewide stay at home orders and are not included. Cluster 1 - states that implemented stay at home orders before March 29th, 2020, and Cluster 2 - states that implemented these orders after March 29th. **(B)** Case rates of lab-confirmed COVID-19 patients at the start of the stay at home orders implemented by each state. Cases rates are the number of cases per 10,000 of the county population. **(C)** Timeline of public health response (non-pharmaceutical interventions) in the states of New York (NY), California (CA), Florida (FL), and Texas (TX). These interventions included screening and testing, a ban on mass gatherings, stay at home orders, requirements for face masks in public locations, and other state-specific measures. In NY contained a one-mile containment effort around hotspot New Rochelle in Westchester County. In FL airport and roadway, screening was implemented for travelers coming to FL from the tri-state region as well as other regions with a high prevalence of COVID-19. In TX Airport and roadway, screening was implemented mainly for travelers coming into TX from the tri-state area and Louisiana, where the prevalence of COVID-19 was high. TX did not enforce mandatory use of cloth facemasks at the state level. Travis (4/13), Harris (4/13), Bexar (4/16), Dallas County (4/18) ordered mandatory facemasks.

Mass Gatherings

The next primary public health intervention implemented across all four states was the cancellation of mass gatherings of 250 individuals, followed by 50 individuals per location (**Supplemental Tables 2**–**5**). These orders followed shortly after initial cases were identified in each state. Events that brought in large amounts of attendance, such as concerts, sporting events, and festivals were canceled first. Next, the states incrementally decreased the number of people allowed to gather in one location until, eventually, the state recommended that people should only interact with those who were within the same household.

Stay at Home Orders

One of the most rigorous measures utilized during COVID-19 was the stay at home orders. California was under stay at home order for 50 days (March 19th to May 7th) (25). The stay at home order in California was implemented more rigorously at the county level because the state-level order acted more as a recommendation (**Supplemental Table 3**). The NY "State on PAUSE" plan stay at home order was enforced for 68 days (March 22nd to May 28th) before the state started its Phase one reopening plan (26–28). Florida state stay at home order was in effect for 27 days (April 3rd to April 29th) (29). Texas implemented a stay at

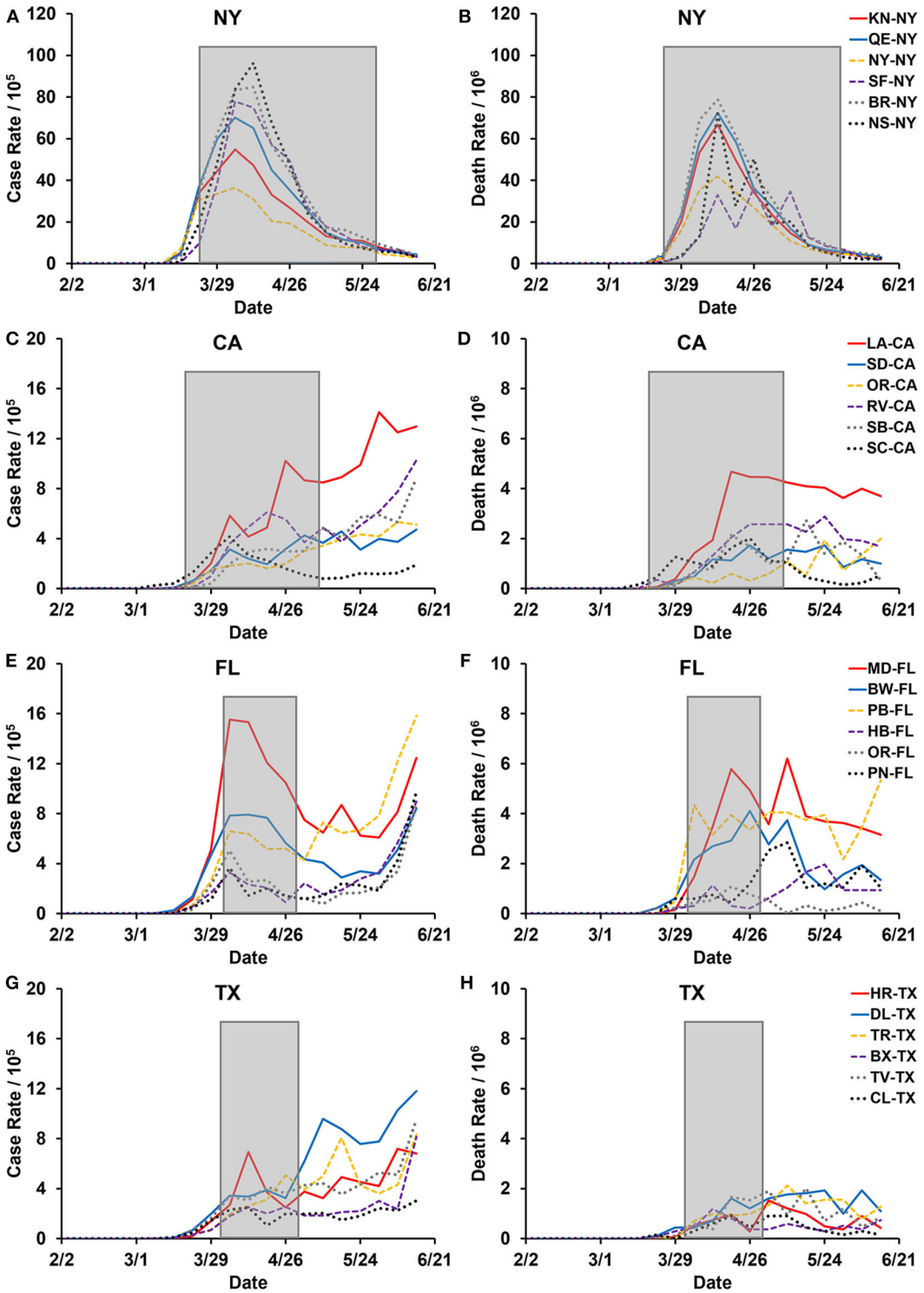


FIGURE 2 | United States COVID-19 cases and mortality in the six most populous counties in the states of New York, California, Florida, and Texas. COVID-19 Cases and deaths are presented as 7-day averages from data provided by Johns Hopkins University and the City of New York (7). Gray boxed areas are the duration statewide stay-at-home orders that were implemented by each state: New York (NY) March 22nd to May 28th (68 days), California (CA) March 19th to May 7th (50 days), Florida (FL) April 3rd to April 29th (27 days), and Texas (TX) April 2nd to April 20th (29 days). **(A,C,E,G)** Case rates are new confirmed COVID-19 cases per 100,000 population in the respective counties. **(B,D,F,H)** Death rates are new COVID-19 related deaths per 1,000,000 population in the individual counties. **(A,B)** Six most populous counties in the state of NY: KN-NY - Kings, QE-NY - Queens, NY-NY - New York, SF-NY - Suffolk, BR-NY - Bronx, and NS-NY - Nassau. **(C,D)** Six

(Continued)

FIGURE 2 | most populous counties in the state of CA: LA-CA - Los Angeles, SD-CA - San Diego, OR-CA - Orange, RV-CA - Riverside, SB-CA - San Bernardino, and SC-CA - Santa Clara. **(E,F)** Six most populous counties in the state of FL: MD-FL - Miami-Dade, BW-FL - Broward, PB-FL - Palm Beach, HB-FL - Hillsborough, OR-FL - Orange, and PN-FL - Pinellas. **(G,H)** Six most populous counties in the state of TX: HR-TX - Harris, DL-TX - Dallas, TR-TX - Tarrant, BX-TX - Bexar, TV-TX - Travis, and CL-TX - Collin.

home order for 29 days before relaxing these measures statewide (April 2nd to April 30th) (30).

Many US states enacted stay at home orders very early on in the COVID-19 transmission. States with early COVID-19 cases placed these measures before April 29th (cluster 1) and did so with a statewide case count of fewer than 2,000 cases, while states that put stay at home orders after April 29th did so before reaching 5,000 cases (cluster 2) (**Figure 1A**, **Supplemental Table 1**). When adjusted to the county population, these measures were implemented with case rates of below 50 cases per 10,000 (**Figure 1B**). The only exception was New York, which implemented these measures after 11,700 cases were confirmed (**Figure 1A**).

Cloth Face Masks

On April 3rd, the Centers for Disease Control and Prevention (CDC) released its recommendation for all individuals to use cloth face masks when in public (31). The goal of this recommendation was to reduce the viral transmission from asymptomatic carriers that may unknowingly spread to disease to susceptible individuals (5, 32). While the extent to which the effectiveness of this measure is debatable, it helps bring more awareness to the public and help curtail the person-to-person transmission of the virus (33). California was the first to implement this statewide on April 1st, which was 2 days before the CDC's recommendation (**Figure 1C**). New York also implemented this measure as a state-level order, but it happened 2 weeks after the CDC's recommendation. Florida and Texas only recommended face coverings at the state-level but was mandated in most counties (**Supplemental Tables 4, 5**).

DIFFERENCES IN STATEWIDE RESPONSES TO COVID-19

The public health interventions implemented across the four states, New York, California, Florida, and Texas, were very similar. Any differences stem from the relative time of implementation and the intensity of measures taken. Unfortunately, New York was one of the first states severely affected by COVID-19 and was likely too late to implement these preventative measures (**Figures 1A,B**, **2B**). The initial wave of COVID-19 in New York, therefore, resembled that of Philadelphia during the 1918 pandemic. California, on the other hand, initiated precautionary measures early and seemed to follow the outcomes of St. Louis, at least in the initial stages (**Figures 2C,D**). Regulations in both of these states were more stringent, and often had consequences such as fines and jail time tied to not adhering to them.

In Texas and Florida, the implementation of specific public health interventions was less rigorous as compared to California and New York. In Texas, for example, the regulations were not implemented as quickly or as firmly at the state-level. Some public health interventions, such as the ban on gathering, stay at home orders, and wearing cloth face masks, may have been perceived as violations of individual liberties and disrupting businesses. In many ways, the small-government philosophy of these states left essential decisions and actions to be made at the county-level. Around the time many states went into shut down mode, spring break activities remained open in Florida. The decision to not shut down before spring break was made in support of the state's economy. It was only after large tourist attractions, including Universal Studios and Disney World, decided to close were more rigorous measures put in place in Florida.

THE SPREAD OF COVID-19 ACROSS STATES AND COUNTIES

During the 1st months of COVID-19, the disease spread rapidly across the United States. In New York, the number of positive cases grew exponentially over the 1st month of the pandemic, especially in the New York City area and surrounding boroughs. However, unlike other states, the number of daily cases in New York has decreased consistently since the end of April. In California, Florida, and Texas, the number of daily cases has continued to increase over time at a slower rate compared to New York. To better understand the dynamics of COVID-19 spread in each of these states, we reviewed the number of cases and deaths in the six most populous counties in each of these states (**Figure 2**).

In New York, the most populous counties all experienced a similar first wave of COVID-19, with a peak of about 100 cases per 10,000 people in early April (**Figure 2A**). Most counties in the state of California continued to have a relatively slow, but steady rise in the number of cases, making it difficult to distinguish between a first and a second wave (**Figure 2C**). We observed a similar pattern in the counties in Florida and Texas, except Miami-Dade County in Florida, which showed a peak case rate of about 15 cases per 10,000 people in early April (**Figures 2C,E,G**). Among these states, it is clear that New York experienced a robust first wave and a negligible second wave of the COVID-19 pandemic. While California, Florida, and Texas were spared from a significant first wave with cases rate peaking at < 20 cases per 10,000, they are now facing a much higher risk for a prolonged second wave of the disease.

US COVID-19 INTERVENTIONS FAILED TO REPLICATE 1918 PANDEMIC OUTCOMES

In the COVID-19 pandemic, the goal of effective public health preventative measures implemented was to mitigate and contain the spread of the disease. In the US, for the most part, public

health interventions followed the principles of effective NPIs. They were implemented early on in the pandemic, using multiple preventative measures, with high intensity and for average durations longer than 45 days (**Figure 1**, **Supplement Table 1**). The exception to this was New York, which delayed the initiation of these measures (**Figures 1A,B**). This caused New York to experience a peak first wave, with hospitals reaching their capacity and a peak number of deaths occurring during mid-April (**Figure 1B**). However, New York enforced its preventative measures for close to 3 months, which in turn helped them bring their daily case rates to < 5 cases per 10,000 by the end of June.

In contrast to New York, most other states followed the evidence-based recommendations, as stated above (**Figure 1**). This helped states "flatten the curve" to various degrees and control the initial spread of COVID-19 within their states. However, these public health interventions seemed to have also prolonged the transmission potential of the COVID-19 as states, including California, Florida, and Texas were experiencing new daily highs in confirmed cases by the end of June 2020 (1). While the general expectation was that US states would follow the outcome of St. Louis during the 1918 pandemic, they have fallen short of replicating this desired outcome. On the contrary, by the end of June 2020, many such states were reimplementing statewide partial shutdown measures to prevent a potential second wave of COVID-19.

DISCUSSION

While the United States failed to prevent the early spread of COVID-19 effectively, some countries had better success containing the Coronavirus with their public health interventions. In Iceland, for example, when cases were identified, public health officials implemented the following strategies: quarantine requirements for international travelers, rigorous tracing of infection, ban on gatherings larger than 20 persons, school closures with limited openings of elementary and preschools, defining areas of higher risk, and regular communication with the general public (34). New Zealand, another island nation with great success, was more rigorous in the process by modifying and intensifying pre-existing plans for the management of influenza pandemics from previous outbreaks (35). These methods included the declaration of a national emergency, a nationwide lockdown, closure of non-essential work locations, banning social gatherings, extreme restrictions on travel, and closure of all schools. Furthermore, as part of this intensified strategy, border security was also tightly regulated. However, there are distinct differences between Iceland, New Zealand, and the United States. Iceland and New Zealand are small island nations with much smaller populations, making it much easier to implement rigorous preventative measures, including better travel restrictions and contact tracing. They were also able to coordinate their public health response more consistently nationwide, unlike the US, which enforced COVID-19 interventions mainly at the state level.

Several factors can help explain why the US was unable to effectively replicate the outcomes of St. Louis vs. Philadelphia during the 1918 flu pandemic. These include (1) the level of adherence to these implemented preventative measures and social behaviors, (2) disparities in social determinants of health, and (3) extensive global and domestic travel with little restrictions. Regardless of the public health intervention intensity, they can be ineffective if people do not consistently adhere to them. Besides, numerous risk factors have been identified for COVID-19 and its clinical outcomes. These include advanced age, sex, immune-compromised status, and comorbidities, including chronic respiratory diseases, diabetes, and hypertension (36–38). American Indians, African Americans, and Hispanic individuals have been reported to be four to five times more likely to be hospitalized for COVID-19 when compared to non-Hispanic whites (39). Disparities in social determinants of health, such as access to healthcare, uninsured population, employment, poverty, education, and population density, can also contribute to the differences observed in COVID-19 transmission. Potential clusters of these risk factors and health determinates present in different geographic regions can lead to the disproportionate spread of the Coronavirus. In conclusion, it is crucial to consider factors such as adherence to preventative measures, and health disparities, in evaluating the effectiveness of COVID-19 interventions implemented. These factors likely caused the US early COVID-19 public health measures to be less effective in containing the Coronavirus pandemic and is an important further direction of research.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

AJ analyzed the US public health response and helped with the figure preparation, writing, and editing of the manuscript. BP aided in intervention comparative analysis and helped with the writing and editing of the manuscript. TG aided in disease spread, comparative analysis, helped with the figure preparation, writing, and editing of the manuscript. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

We thank Drs. Richard S. Gunasekera, Genti Buzi, and Biola research students JongWon See, Alexis M. Gulsvig, and Sumaia G. Khoury for their discussions and data analysis on this research topic. We also thank Dr. Jeffrey S. Wang, Infectious Disease Specialist at Kaiser Permanente, Anaheim, California, for his clinical insights.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2020.579559/full#supplementary-material

REFERENCES

1. CDC. *Coronavirus Disease 2019 (COVID-19) Cases in the U.S*. Centers for Disease Control and Prevention. (2020) Available online at: https://www.cdc.gov/coronavirus/2019-ncov/cases-updates/cases-in-us.html (accessed August 10, 2020).
2. Hogan GL. *Governor Larry Hogan Declares State of Emergency, Expands Statewide Response to Novel Coronavirus. Office of Governor*. (2020). Available online at: https://governor.maryland.gov/2020/03/05/governor-larry-hogan-declares-state-of-emergency-expands-statewide-response-to-novel-coronavirus/ (accessed August 10, 2020).
3. Inslee GJ. *Inslee Issues COVID-19 Emergency Proclamation. Office of Governor*. (2020). Available online at: https://www.governor.wa.gov/news-media/inslee-issues-covid-19-emergency-proclamation (accessed August 10, 2020).
4. Newsom GG. *Governor Newsom Declares State of Emergency to Help State Prepare for Broader Spread of COVID-19*. Office of Governor. (2020). Available online at: https://www.gov.ca.gov/2020/03/04/governor-newsom-declares-state-of-emergency-to-help-state-prepare-for-broader-spread-of-covid-19/ (accessed August 10, 2020).
5. Galbadage T, Peterson BM, Gunasekera RS. Does COVID-19 spread through droplets alone? *Front Public Health*. (2020) 8:163. doi: 10.3389/fpubh.2020.00163
6. Wilder-Smith A, Freedman DO. Isolation, quarantine, social distancing and community containment: pivotal role for old-style public health measures in the novel coronavirus (2019-nCoV) outbreak. *J Travel Med*. (2020) 27:taaa020. doi: 10.1093/jtm/taaa020
7. Dong E, Du H, Gardner L. An interactive web-based dashboard to track COVID-19 in real time. *Lancet Infect Dis*. (2020) 20:533–4. doi: 10.1016/s1473-3099(20)30120-1
8. Mills CE, Robins JM, Lipsitch M. Transmissibility of 1918 pandemic influenza. *Nature*. (2004) 432:904–6. doi: 10.1038/nature03063
9. Ferguson NM, Cummings DA, Cauchemez S, Fraser C, Riley S, Meeyai A, et al. Strategies for containing an emerging influenza pandemic in Southeast Asia. *Nature*. (2005) 437:209–14. doi: 10.1038/nature04017
10. Markel H, Stern AM, Navarro JA, Michalsen JR, Monto AS, DiGiovanni C. Nonpharmaceutical influenza mitigation strategies, US communities, 1918-1920 pandemic. *Emerg Infect Dis*. (2006) 12:1961–4. doi: 10.3201/eid1212.060506
11. Ferguson NM, Cummings DA, Fraser C, Cajka JC, Cooley PC, Burke DS. Strategies for mitigating an influenza pandemic. *Nature*. (2006) 442:448–52. doi: 10.1038/nature04795
12. Bootsma MC, Ferguson NM. The effect of public health measures on the 1918 influenza pandemic in U.S. cities. *Proc Natl Acad Sci USA*. (2007) 104:7588–93. doi: 10.1073/pnas.0611071104
13. Handel A, Longini IM Jr, Antia R. What is the best control strategy for multiple infectious disease outbreaks? *Proc Biol Sci*. (2007) 274:833–7. doi: 10.1098/rspb.2006.0015
14. Hatchett RJ, Mecher CE, Lipsitch M. Public health interventions and epidemic intensity during the 1918 influenza pandemic. *Proc Natl Acad Sci USA*. (2007) 104:7582–7. doi: 10.1073/pnas.0610941104
15. Kalnins I. The Spanish influenza of 1918 in St. Louis, Missouri. *Pub Health Nurs*. (2006) 23:479–83. doi: 10.1111/j.1525-1446.2006.00586.x
16. Gupta S, Nguyen TD, Rojas FL, Raman S, Lee B, Bento A, et al. *Tracking Public and Private Responses to the COVID-19 Epidemic: Evidence from State and Local Government Actions*. National Bureau of Economic Research. (2020).
17. CDC. *Public Health Screening to Begin at 3 U.S. Airports for 2019 Novel Coronavirus ("2019-nCoV")*. Centers for Disease Control and Prevention. (2020). Available online at: https://www.cdc.gov/media/releases/2020/p0117-coronavirus-screening.html (accessed August 10, 2020).
18. Florida Department of Health. *Clinical Screening Tool for Identifying Persons Under Investigation*. (2020). Available online at: https://floridahealthcovid19.gov/health-care-providers/ (accessed August 10, 2020).
19. State of California. *Testing and Treatment*. (2020). Available online at: https://covid19.ca.gov/testing-and-treatment/ (accessed August 10, 2020).
20. State of New York. *COVID-19 Testing*. (2020). Available online at: https://coronavirus.health.ny.gov/covid-19-testing (accessed August 10, 2020).
21. Texas Department of State Health Services. *COVID-19 Testing Information*. (2020). Available online at: https://www.dshs.state.tx.us/coronavirus/testing.aspx (accessed August 10, 2020).
22. City of New York. *NYC COVID-19 Citywide Information Portal*. (2020). Available online at: https://www1.nyc.gov/site/coronavirus/get-tested/covid-19-testing.page (accessed August 10, 2020).
23. Florida Division of Emergency Management. *Florida COVID-19 Mobile Testing Lab*. (2020). Available online at: https://flcovidtest-floridadisaster.hub.arcgis.com/ (accessed August 10, 2020).
24. CDC. *Coronavirus Disease 2019 (COVID-19) COVIDTracer 1.0*. Centers for Disease Control and Prevention. (2020). Available online at: https://www.cdc.gov/coronavirus/2019-ncov/php/contact-tracing/COVIDTracer.html (accessed August 10, 2020).
25. State of California. *Stay Home Q&A*. (2020). Available online at: https://covid19.ca.gov/stay-home-except-for-essential-needs/ (accessed August 10, 2020).
26. Cuomo GAM. *No. 202.31: Continuing Temporary Suspension and Modification of Laws Relating to the Disaster Emergency*. (2020). Available online at: https://www.governor.ny.gov/news/no-20231-continuing-temporary-suspension-and-modification-laws-relating-disaster-emergency (accessed August 10, 2020).
27. State of New York. *New York State on PAUSE*. (2020). Available online at: https://coronavirus.health.ny.gov/new-york-state-pause (accessed August 10, 2020).
28. State of New York. *Reopening New York*. (2020). Available online at: https://forward.ny.gov/ (accessed August 10, 2020).
29. State of Florida. *Executive Order Number 20-91 (Essential Services and Activities During COVID-19 Emergency)*. (2020). Available online at: https://www.flgov.com/wp-content/uploads/orders/2020/EO 20-91-compressed.pdf (accessed August 10, 2020).
30. Abbott GG. *Executive Order GA14 (Relating to Statewide Continuity of Essential Services Aizd Activities During the COVID-19 Disaster)*. (2020). Available online at: https://lrl.texas.gov/scanned/govdocs/Greg%20Abbott/2020/GA-14.pdf (accessed August 10, 2020).
31. CDC. *Considerations for Wearing Cloth Face Coverings*. Centers for Disease Control and Prevention. (2020). Available online at: https://www.cdc.gov/coronavirus/2019-ncov/prevent-getting-sick/cloth-face-cover-guidance.html (accessed August 10, 2020).
32. Esposito S, Principi N, Leung CC, Migliori GB. Universal use of face masks for success against COVID-19: evidence and implications for prevention policies. *Eur Respir J*. (2020) 55:2001260. doi: 10.1183/13993003.01260-2020
33. Eikenberry SE, Mancuso M, Iboi E, Phan T, Eikenberry K, Kuang Y, et al. To mask or not to mask: modeling the potential for face mask use by the general public to curtail the COVID-19 pandemic. *Infect Dis Model*. (2020) 5:293–308. doi: 10.1016/j.idm.2020.04.001
34. Iceland Directorate of Health. *Iceland's Response: Government objectives and actions*. (2020). Available online at: https://www.covid.is/sub-categories/icelands-response (accessed August 10, 2020).
35. Baker MG, Kvalsvig A, Verrall AJ. New Zealand's COVID-19 elimination strategy. *Med J Aust*. (2020). doi: 10.5694/mja2.50735. [Epub ahead of print].
36. Galbadage T, Peterson BM, Awada J, Buck AS, Ramirez DA, Wilson J, et al. Systematic review and meta-analysis of sex-specific COVID-19 clinical outcomes. *Front Med*. (2020) 7:348. doi: 10.3389/fmed.2020.00348
37. Li LQ, Huang T, Wang YQ, Wang ZP, Liang Y, Huang TB, et al. COVID-19 patients' clinical characteristics, discharge rate, and fatality

rate of meta-analysis. *J Med Virol.* (2020) 92:577–83. doi: 10.1002/jmv.25757

38. Richardson S, Hirsch JS, Narasimhan M, Crawford JM, McGinn T, Davidson KW, et al. Presenting characteristics, comorbidities, and outcomes among 5700 patients hospitalized with COVID-19 in the New York city area. *JAMA.* (2020) 323:2052–9. doi: 10.1001/jama.2020.6775
39. CDC. *COVID-19 in Racial and Ethnic Minority Groups.* (2020). *Centers for Disease Control and Prevention.* Available online at: https://www.cdc.gov/coronavirus/2019-ncov/need-extra-precautions/racial-ethnic-minorities.html (accessed August 10, 2020).

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Medicine

REVIEW
published: 15 September 2020
doi: 10.3389/fmed.2020.00546

Therapeutic Options Against the New Coronavirus: Updated Clinical and Laboratory Evidences

Amélia Carolina Lopes Fernandes[1], *Adson José Martins Vale*[2,3], *Fausto Pierdoná Guzen*[3], *Francisco Irochima Pinheiro*[3,4], *Ricardo Ney Cobucci*[3,4] *and Eduardo Pereira de Azevedo*[3]*

[1] Nurse Department, Nurse School, Universidade do Estado do Rio Grande do Norte (UERN), Mossoró, Brazil,
[2] Tocogynecology Department, Medical School, Universidade Federal do Rio Grande do Norte (UFRN), Natal, Brazil,
[3] Graduate Program of Biotechnology, Laureate International Universities - Universidade Potiguar (UnP), Natal, Brazil,
[4] Medical School, Laureate International Universities - Universidade Potiguar (UnP), Natal, Brazil

The pandemic caused by the new coronavirus (SARS-Cov-2) has encouraged numerous *in vitro* studies and clinical trials around the world, with research groups testing existing drugs, novel drug candidates and vaccines that can prevent or treat infection caused by this virus. The urgency for an effective therapy is justified by the easy and fast viral transmission and the high number of patients with severe respiratory distress syndrome who have increasingly occupied intensive care hospital beds, leading to a collapse in health systems in several countries. However, to date, there is no sufficient evidence of the effectiveness of any researched therapy. The off-label or compassionate use of some drugs by health professionals is a reality in all continents, whose permission by regulatory agencies has been based on the results of some clinical trials. In order to guide decision-making for the treatment of COVID-19, this review aims to present studies and guidelines on the main therapies that have been and are currently being tested against SARS-CoV-2 and to critically analyze the reported evidences.

Keywords: COVID-19, SARS-CoV-2, coronavirus, drug treatment, prophylaxis, viral infection

OPEN ACCESS

Edited by:
Zisis Kozlakidis, International Agency for Research on Cancer (IARC), France

Reviewed by:
Javier Carbajo-Lozoya, Technical University of Munich, Germany
Charles Narh, Burnet Institute for Medical Research, Australia

***Correspondence:**
Eduardo Pereira de Azevedo
eduardo.azevedo@unp.br

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: *22 June 2020*
Accepted: *31 July 2020*
Published: *15 September 2020*

Citation:
Fernandes ACL, Vale AJM, Guzen FP, Pinheiro FI, Cobucci RN and de Azevedo EP (2020) Therapeutic Options Against the New Coronavirus: Updated Clinical and Laboratory Evidences. Front. Med. 7:546. doi: 10.3389/fmed.2020.00546

INTRODUCTION

The new coronavirus (SARS-CoV-2) is an RNA virus that belongs to the Coronaviridae family and to the Nidovirales order. It belongs to the same beta subgroup of viruses that caused severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV) in the past decades, sharing 80% and 50% of their genome, respectively (1). Since the first cases of the novel coronavirus disease (COVID-19) reported in Wuhan (China) in late 2019, more than 80,000 cases have been reported in China alone. According to the World Health Organization (WHO), the epidemic COVID-19 peaked between late January and early February 2020 in China and the rate of new cases declined substantially in early March (2). A World Health Organization report published in March estimated a global mortality rate of 3.4% (3) and until July 1, 2020, 10,357,662 confirmed cases were registered, with 508,055 deaths (4).

In most of the cases, the disease caused by SARS-CoV-2 is represented by a mild upper airway infection with symptoms that include fever, cough, sore throat, shortness of breath, loss of smell and taste, as well as diarrhea (5). However, it can progress to severe acute respiratory syndrome in a short period of time. The virus usually infects the type 2 alveolar cells in the lung, which may explain the severe alveolar damage found in cases of SARS-CoV-2. Due to the astonishing number

of COVID-19 patients requiring hospitalization and mechanical ventilation, this current pandemic has already strained healthcare systems in several countries (6).

To date, multiple therapies have been proposed based mostly on the findings of *in vitro* studies and on observational and clinical trials. In these studies, researchers investigated the efficacy and safety of new and old drugs by studying their potential in inhibiting the entry and fusion of the virus within the cells, in controlling viral replication, in suppressing the intense inflammatory response and in controlling hypercoagulability (6–8). In a recent review, Sanders et al. presented a panel of articles published in English that focused on the treatment of adults with COVID-19. The authors admitted that the growing number of publications on therapies against this virus indicates that discoveries about such therapies are constantly evolving (9).

Although no effective vaccine or drug has been approved to treat COVID-19 until the date of writing this paper, some clinical trials have been carried out with already approved drugs, as well as with vitamins and biological samples with promising effectiveness. The aim of this work is to review the literature about which therapies are being researched against the new coronavirus, update the data published in previous reviews and critically evaluate the evidence from the *in vitro* and *in vivo* studies.

METHOD

For this review, the inclusion criteria were guidelines as well as clinical, *in vivo* and *in vitro* studies that investigated the use of drugs, chemicals, vitamins and biological agents, with reported efficacy and adverse effects, intended for COVID-19 prophylactic and/or therapeutic purposes.

Guidelines and articles published until July 20th, 2020 were searched without language restriction in Pubmed, Embase, Scopus, and Up ToDate databases. Search terms included coronavirus, severe acute respiratory syndrome coronavirus 2, 2019-nCoV, SARS-CoV-2, COVID-19 in combination with therapeutics, therapy, treatment, *in vitro*, drug evaluation studies, cohort studies, clinical trials, guidelines and pharmacology. The search resulted in a total of 3,948 articles. The authors independently reviewed the titles and abstracts for inclusion. Additional relevant articles were identified based on the citations and references of each paper.

The drugs are presented in sections arranged in alphabetical order, with the critical analysis of the evidences being individually presented. A summary of the selected studies, researched drugs and dosage regimen is presented in **Table 1**.

DRUGS AND PERSPECTIVES

Anticoagulants

Recent studies (11, 24) have demonstrated that patients infected with SARS-CoV-2 who have progressed to viral pneumonia with severe respiratory distress syndrome were diagnosed with disseminated intravascular coagulation and presented abnormal coagulation results during the later stages of the disease. In those infected, increased concentrations of D-dimer and other fibrin degradation products were associated with poor prognosis. Fibrinolysis and pulmonary coagulation are believed to be regulated by several pro-inflammatory cytokines, however, the concrete mechanisms for coagulopathy have not been identified yet (28, 29).

Infection-induced endothelial cell dysfunction results in increased production of thrombin which might lead to a state of hypercoagulability. In addition, hypoxia resulting from severe viral pneumonia can stimulate thrombosis due to increased blood viscosity (28). Ozolina et al. (30) demonstrated that the plasma concentrations of tissue factor and plasminogen activator inhibitor-1 were significantly higher in patients with severe respiratory distress syndrome (SARS) in comparison with those without the syndrome (**Figure 1**).

A recent review reported studies that compared the D-dimer and fibrin values among patients with COVID-19. The authors point out that coagulopathy seems to be related to the severity of illness and to the extension of the inflammatory process and not to the intrinsic viral activity. In addition, they stated that elevated D-dimer at hospital admission has been associated with increased mortality (31).

A meta-analysis (32) including 9 randomized controlled trials and 465 patients with SARS showed that among those treated with low molecular weight heparin, a significant reduction in mortality was observed. Thus, Chinese clinicians decided to use anticoagulants in patients with SARS-CoV-2 as they believed that such drugs could significantly reduce mortality. Seffer et al. (33) claim that viruses bind to immobilized heparin in a similar way as heparan sulfate interacts with the cell surface. This binding is non-reversible and as such, the pathogens are removed from the bloodstream. Thus, they conclude that since heparin has already shown to be effective in reducing viral load in animal models infected by Zica virus, cytomegalovirus, adenovirus and SARS-CoV-2, additional clinical trials are need in order to prove its effectiveness in the treatment of patients with COVID-19.

Tang et al. (18) made a retrospective survey of 449 patients (268 men) aged $\geq$18 that were hospitalized in China due to serious respiratory problems (respiratory rate $>$ 30/min, arterial oxygen saturation $\leq$ 93% at rest and $PaO_2/FiO_2 \leq 300$ mmHg) as a result of COVID-19. Of these, 99 received heparin, mainly low molecular weight (94 received enoxaparin. 40–60 mg per day) for at least 7 days. According to the authors, the early use of anticoagulant therapy in severe cases of COVID-19 was suggested in China based on the analogy with what is known to occur in other viral infections. This hypothesis has been corroborated by a recent evidence of occlusion necropsy and formation of microthrombi in small pulmonary vessels in those infected with the new coronavirus. This study demonstrated that 28-day mortality was no different between heparin users and non-users (30.3 and 29.7%, respectively), but was significantly lower (40.0 vs. 64.2%, $p = 0.029$) in the group of heparin users with severe coagulopathy induced by sepsis and also in those with D-dimer $>$ 6 times the upper limit of normal (32.8 vs. 52.4%, $p = 0.017$). The authors suggest that only patients with more severe forms of COVID-19 (those with considerably

TABLE 1 | Summary of the drugs and vitamins that have been investigated for prophylaxis and treatment of COVID-19 infection with their respective recommended dose and posology.

Author (Reference)	Study design	Drugs	Dose and posology
Yao et al. (10)	*In-vitro*	Hydroxychloroquine and chloroquine	Hydroxychloroquine – 400 mg, twice daily, followed by 200 mg twice daily for 4 days Chloroquine – 500 mg twice daily 5 days
Huang et al. (11)	Randomized clinical trial	Chloroquine, lopinavir, and ritonavir	Chloroquine – 500 mg twice daily 10 days. Lopinavir/Ritonavir 400/100 mg, twice daily, for 10 days
Gautret et al. (12)	Open label non-randomized clinical trial	Hydroxychloroquine and azithromycin	Hydroxychloroquine – 600 mg daily, followed by 200 mg twice daily for 10 days Azithromycin – 500 mg on day one, followed by 250 mg per day for 04 days
Lagier et al. (13)	Cross-sectional	Hydroxychloroquine and azithromycin	Hydroxychloroquine – 200 mg three times daily for 10 days Azithromycin – 500 mg on day one, followed by 250 mg per day for 4 days
Mitjá et al. (14)	Randomized clinical trial	Hydroxychloroquine	Hydroxychloroquine – 800 mg on day1, followed by 400 mg once daily for 6 days
Skipper et al. (15)	Randomized clinical trial	Hydroxychloroquine	Hydroxychloroquine – 800 mg on day1, followed by 600 mg once daily for 5 days
Cavalcanti et al. (16)	Randomized clinical trial	Hydroxychloroquine and Azithromycin	Hydroxychloroquine at a dose of 400 mg twice daily plus azithromycin at a dose of 500 mg once daily for 7 days
Borba et al. (17)	Randomized clinical trial	Chloroquine	Chloroquine – 600 mg twice daily for 10 days Chloroquine – 450 mg for 5 days, twice daily only on the first day
Tang et al. (18)	Cross-sectional	Enoxaparin	Enoxaparin– 40–60 mg per day for at least 7 days
Duan et al. (19)	Cross-sectional	Convalescent plasma	Convalescent plasma– 200 ml single dose
Health Alert Network (20)	Guidelines	Interferon-alpha (IFN-α); lopinavir/ritonavir	Interferon-alpha (IFN-α) in 5.000U twice a day (bis in die – BID); Lopinavir/ritonavir (400/100mg twice a day through oral route)
Wang et al. (21)	Cohort	Favipiravir + oseltamivir	Favipiravir 1,600 mg BD on day 1 and 800 mg BD on 2–10 days + Oseltamivir 75 mg BD once a day for 10 days
Goldman et al. (22)	Randomized	Remdesivir	Remdesivir 200 mg intravenous on day 1 and 100 mg for 9 days Or Remdesivir 200 mg intravenous on day 1 and 100 mg for 5 days
Wang et al. (23)	Randomized double-blind Controlled Multicentric Trial	Remdesivir	Remdesivir 200 mg intravenous on day 1 and 100 mg for 9 days
Chen et al. (24)	Randomized clinical trial	Oseltamivir Ganciclovir Lopinavir/ritonavir	Oseltamivir 75 mg twice a day through oral route Ganciclovir 0.25 mg twice a day intravenous Lopinavir/ritonavir 500mg twice a day, oral route
Caly et al. (25)	*In vitro* controlled trial	Ivermectin	5 μM No correlation with human dose
Rossignol (26)	Clinical trial	Nitazoxanide + Hydroxychloroquine; Hydroxychloroquine	Nitazoxanide 500 mg + Hydroxychloroquine 200 mg twice a day for 10 days; Hydroxychloroquine 200 mg twice a day for 10 days
Grant et al. (27)	Review based on several clinical trials	Vitamin D	Daily dose of 10,000 IU of vitamin D3 for a few weeks and once the levels of 25(OH)D increases, the daily dose should decrease to 5,000 IU

high D-dimer) may benefit from anticoagulant treatment, especially with low molecular weight heparin (**Figure 1**). However, they claim that such findings need to be confirmed by prospective studies.

Although the study of Tang et al. is the only one so far that investigated the use of anticoagulant therapy in patients infected with SARS-CoV-2, the International Society of Thrombosis and Haemostasis (ISTH) recently published a protocol on the management of coagulopathy. In this document, the recommendation is that treatment with low molecular weight heparin (enoxaparin 40–60 mg/day) should be considered for all patients infected with the new coronavirus receiving hospital care, even the patients with mild infection, except for those with active bleeding and platelets below 25,000. The authors stated that there is an additional benefit for using low molecular weight heparin due to its anti-inflammatory properties, which might contribute to a reduction of pro-inflammatory cytokines and may prevent the disseminated intravascular coagulation, which is so common in patients with sepsis (34).

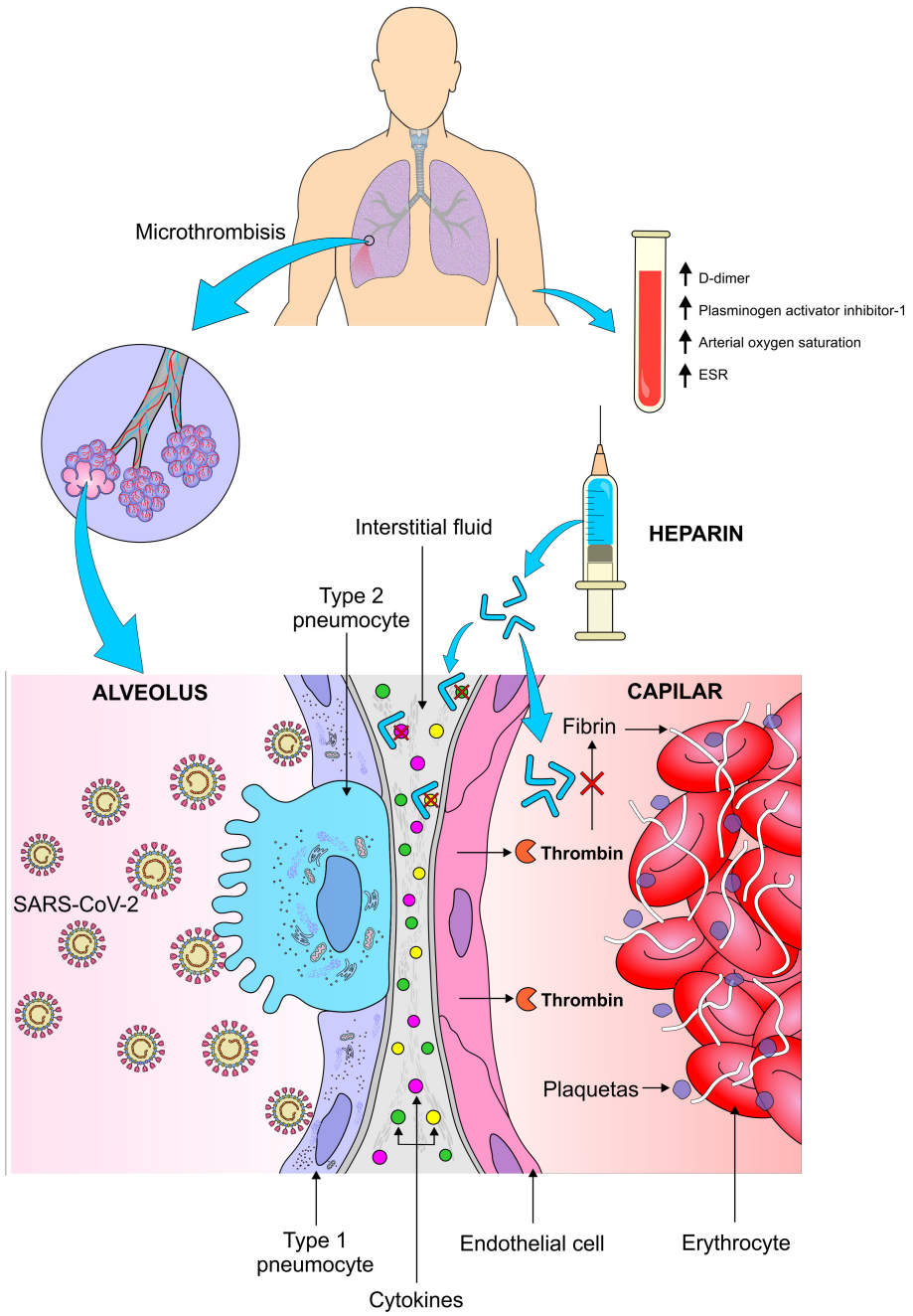


FIGURE 1 | Thromboembolic complications in patients with pulmonary infection caused by SARS-CoV-2 and the mechanism of action of heparin in the pulmonary microthrombotic events. This viral infection results in high levels of cytokines in the pulmonary interstitial fluid, in addition to increased production of thrombin by the pulmonary endothelium, which increases the thromboembolic events in the lung tissue resulting in less oxygenation. The use of heparin reduces the conversion of thrombin to fibrin and decreases the activity of cytokines in the pulmonary interstitium. TSE, Erythematous sedimentation rate. *Figure source: Authors' own drawing*.

Anti-inflammatories

Immune mediators such as inflammatory cytokines and chemokines, including interleukins (IL-1β; IL-6, IL-7, IL-8, IL-9, IL-10), induced protein 10 (IP10), C-reactive protein, tumor necrosis factor (TNF-α), monocyte chemoattracting protein 1 (MCP-1), are significantly elevated in patients with COVID-19. The presence of these mediators was more commonly observed in critically ill patients, in addition to very low levels of lymphocytes in peripheral blood, especially natural Killer cells (NK), which demonstrate that the immunological status is closely related to the prognosis of the disease (**Figure 2**) (35–37).

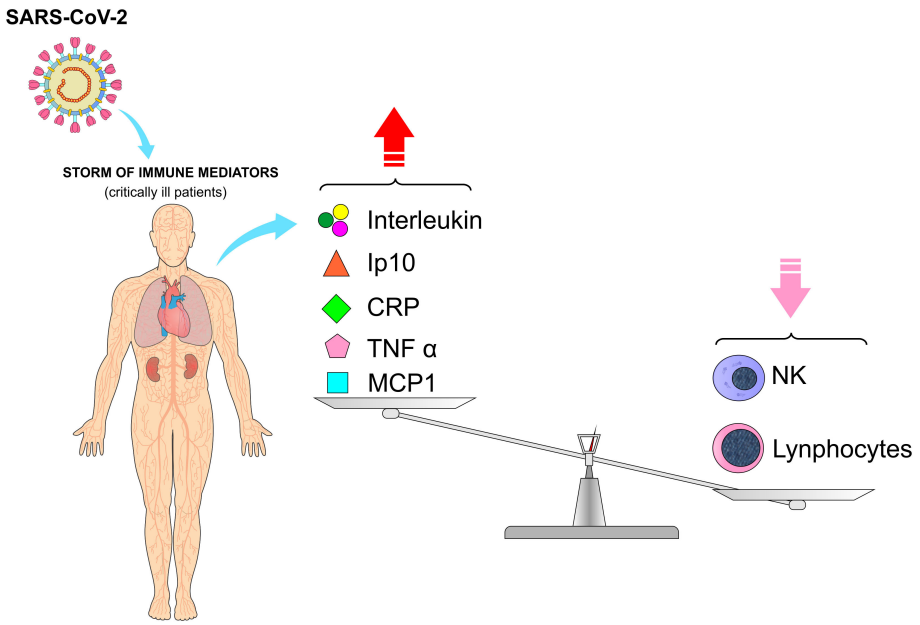


FIGURE 2 | Cytokine release syndrome (CRS) and low levels of lymphocytes in peripheral blood, especially natural Killer cells (NK), in patients infected with SARS-CoV-2. The storm of immune mediators due to infection by SARS-CoV-2 has a direct impact on laboratory tests, with an increase in interleukin, Ip10, CRP, TNT-α, and MCP-1 and a decrease in some of the blood elements such as lymphocytes and NK cells. (Ip10, induced protein 10; CRP, C-reactive protein; TNF-α, tumor necrosis factor; MCP-1, monocyte chemoattracting protein 1). *Figure source: Authors' own drawing*.

In patients with COVID-19 with elevated inflammatory cytokines, the postmortem pathology revealed tissue necrosis, interstitial macrophages and monocyte infiltrations in the lung, heart and gastrointestinal mucosa. In addition, severe lymphopenia with hyperactivated proinflammatory T cells and decreased regulatory T cells are commonly seen in critically ill patients (10, 37, 38). Huang et al. measured the cytokine levels in 41 patients with COVID-19 and the results showed that significantly higher cytokine levels are observed in critically ill patients in different age groups in the presence or absence of comorbidities, as mentioned in other studies. Most critically ill patients with COVID-19 have a considerably high and persistent levels of erythrocyte sedimentation rate and immune mediators, being associated with acute respiratory failure syndrome, hypercoagulation and disseminated intravascular coagulation, manifested as thrombosis, thrombocytopenia and gangrene of extremities. It seems that the immune response worsens lung damage and leads to further complications (10, 36, 38–40).

These findings reveal that patients with COVID-19 are usually accompanied by increased immunological factors with inflammatory responses, justifying that the concentrations of immunological factors are associated with the severity of the disease. In fact, a storm of immune mediators is one of the clinical manifestations of COVID-19. These immune mediators act as pro-inflammatory agents, resulting in the cytokine release syndrome (CRS), being an important factor in the pathology of COVID-19 (39).

Corticosteroids were widely used during outbreaks of severe SARS-CoV1 and MERS-CoV2 and their use are now being considered in patients with COVID-19 in combination with other therapies (11). Corticosteroids have a good inhibitory effect on inflammatory factors and are often used as an auxiliary treatment for viral pneumonia, which is one of the reasons corticosteroids are being commonly prescribed for the treatment of patients with COVID-19 in the intensive care unit. Patients with COVID-19 are treated mainly with symptomatic therapy, however, corticosteroids are widely used in the symptomatic treatment of severe pneumonia (41, 42).

The main anti-inflammatory effect of glucocorticoids involves the inhibition of a high number of pro-inflammatory genes that encode cytokines, chemokines, cell adhesion molecules, inflammatory enzymes and receptors that ultimately address the inflammatory process (42). The use of glucocorticoids can improve early fever, promote the absorption of pneumonia and induce better oxygenation of the airways. However, some studies have shown no beneficial effect of glucocorticoids due to their adverse reactions and delay in eliminating the virus (43).

As described in the Chinese guidelines of COVID-19 (44), clinicians need to be cautious about steroid use due to its nebulous benefits in the scenario of viral respiratory infection. Several studies have reported inferior results in patients with SARS treated with corticosteroids (45) and, in the case of MERS-CoV coronavirus, the results showed that patients who received corticosteroids were more likely to need mechanical ventilation, vasopressors and renal replacement therapy (46). Another concern of corticosteroid use is their short- and long-term adverse effects that may lead to consequences such as joint pain and bone marrow abnormalities in patients with SARS (47).

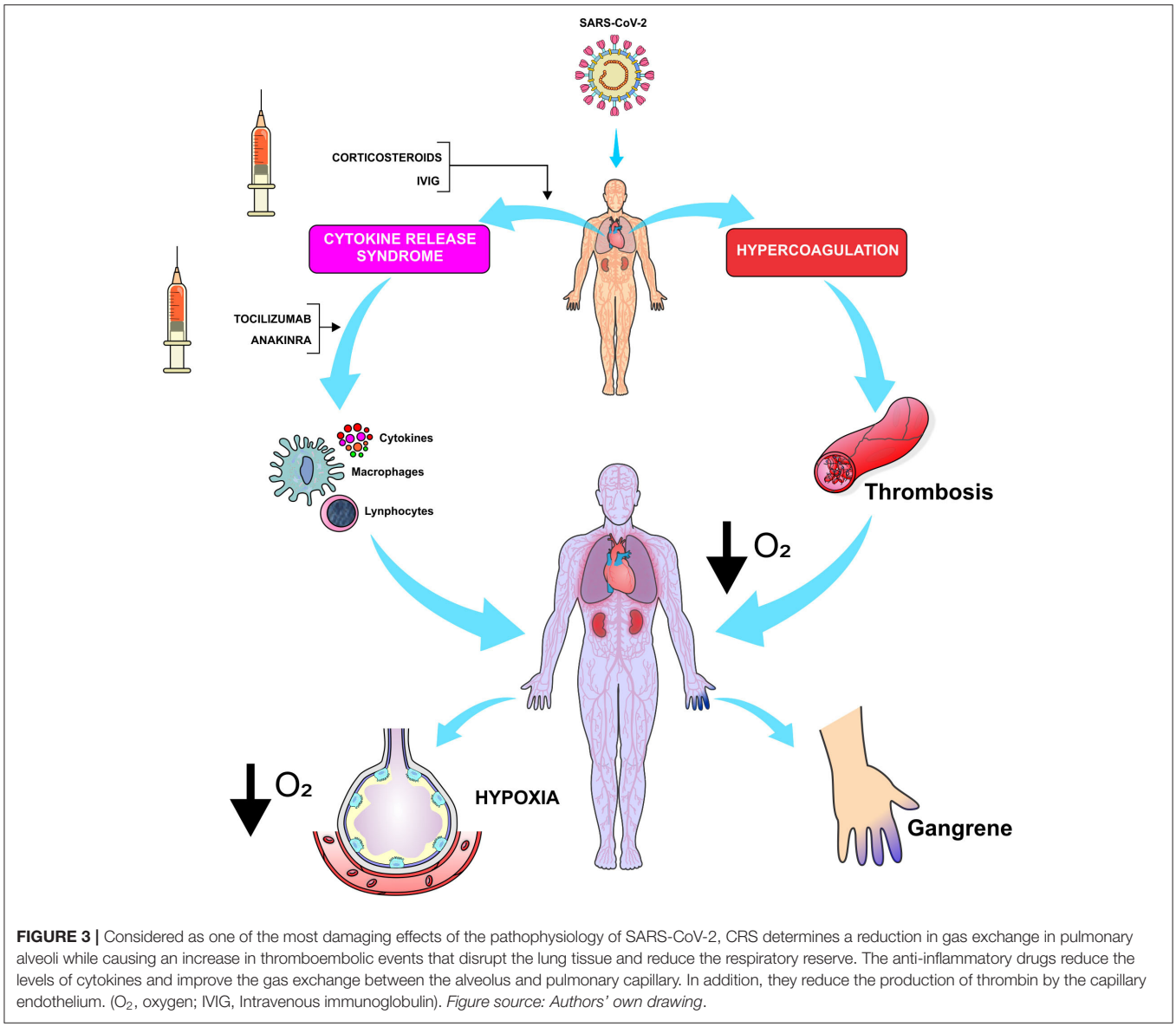


FIGURE 3 | Considered as one of the most damaging effects of the pathophysiology of SARS-CoV-2, CRS determines a reduction in gas exchange in pulmonary alveoli while causing an increase in thromboembolic events that disrupt the lung tissue and reduce the respiratory reserve. The anti-inflammatory drugs reduce the levels of cytokines and improve the gas exchange between the alveolus and pulmonary capillary. In addition, they reduce the production of thrombin by the capillary endothelium. (O_2, oxygen; IVIG, Intravenous immunoglobulin). *Figure source: Authors' own drawing*.

Previous reports have shown that the use of corticosteroids can lead to prolonged removal of viral RNA from the airways (46), blood (48), and feces (49), resulting in longer hospitalization and ultimately increased mortality risk. The main concern is that corticosteroids may delay the elimination of the virus and increase the risk of secondary infection, especially in those with compromised immune system. In addition, biological agents targeting pro-inflammatory cytokines can only inhibit specific inflammatory factors and, therefore, may not be so effective for COVID-19 treatment in which other cytokines may be involved.

Siddiqi and Mehra (50) suggested that the target therapy in stage III of COVID-19 requires the use of immunomodulatory agents to reduce systemic inflammation. At this stage, the use of corticosteroids may be justified if combined with cytokine inhibitors, such as tocilizumab (IL-6 inhibitor) or anakinra (IL-1 receptor antagonist). Intravenous immunoglobulin (IVIG) can also play a role in modulating immune systems under hyper-inflammatory state (**Figure 3**). In general, the prognosis and recovery from this critical stage of the disease are poor and the rapid introduction of this therapy might result in some benefits (36, 50).

Wang et al. reported that 44.9% of patients with COVID-19 have received glucocorticoid therapy with no effective results (42). Russell et al. reported clinical evidences that did not support the treatment with corticosteroids in lung injury caused by COVID-19 (45). Due to the lack of enough evidences, the WHO provisional guidelines (February 22, 2020) do not support the use of systemic corticosteroids in the treatment of viral pneumonia and in suspected cases of COVID-19 (51). Therefore, the efficacy

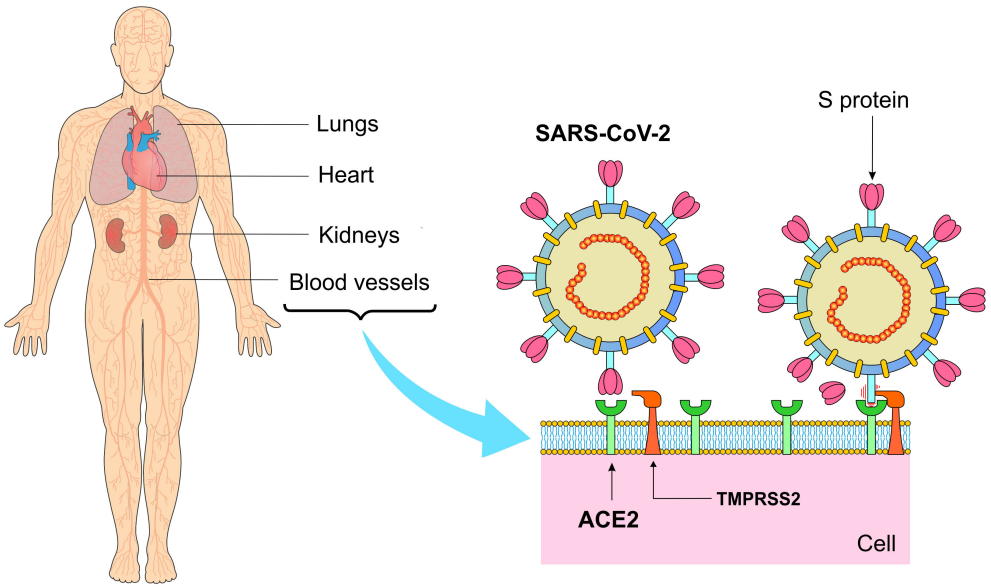


FIGURE 4 | Main sites of angiotensin-converting enzyme 2 (ACE2) expression and binding of S protein to the ACE2 receptor after activation of the transmembrane serine protease 2 (TMPRESS2). ACE2 is a surface enzyme that acts as a port of entry of SARS-CoV-2 into the cells. This enzyme is present mainly in the pulmonary, cardiac, renal and vascular tissues, even though its presence in the adipose tissue places obesity as one of the risk factors for COVID-19. *Figure source: Authors' own drawing*.

and adverse effects associated with the use of glucocorticoids in COVID-19 need to be elucidated.

A review of treatments for acute respiratory distress syndrome based on six studies with a total of 574 patients concluded that there is insufficient evidence to recommend treatment with corticosteroids. Observational data suggest increased mortality and higher secondary infection rates in influenza, as well as impaired clearance of SARS-CoV and MERS-CoV. Patients who received corticosteroid therapy were more likely to develop bacterial infection due to immunosuppression. This can worsen the disease and might lead to death (52, 53).

A team of frontline clinicians in China recommended administration of corticosteroids in low to moderate doses for a short period of time in critically ill patients with COVID-19 pneumonia (54). However, current WHO provisional guidelines (released on January 28, 2020) on the clinical treatment of severe acute respiratory infection in suspected cases of new coronavirus (SARS-CoV-2) advises against the use of corticosteroids, unless otherwise strictly indicated (51).

Other anti-inflammatory drugs, such as baricitinib, also block the production of IFN-γ, which is necessary for fighting the virus, and theoretically may not be suitable for the treatment of the inflammatory response caused by COVID-19. The time frame of anti-inflammatory treatment is very important and according to reports, critically ill patients generally experience abrupt deterioration within 1–2 weeks, which means that the immediate start of anti-inflammatory therapy in this extremely short time window is likely to achieve a favorable response (10, 52).

The receptors for SARS-CoV-2 may be ACE2, which is a cell surface protein that exists widely in cells of the heart, kidney, blood vessels and especially in alveolar epithelial cells. SARS-CoV-2 can invade and enter these cells through endocytosis (**Figure 4**). One of the regulators of endocytosis is protein kinase 1 associated with AP2 (AAK1). AAK1 inhibitors can stop the virus from passing into cells and can be useful in preventing virus infections. Baricitinib, a JAK and AAK1 inhibitor, has been suggested as a possible candidate for the treatment of COVID-19, considering its relative safety and high affinity for ACE2. Therapeutic doses in the range of 2–4 mg once a day is enough to reach the plasma inhibitory concentration (52). However, as mentioned above, the biggest concern with JAK inhibitors is that it can inhibit a variety of inflammatory cytokines, including interferon, which plays an important role in controlling virus activity.

Tocilizumab (TCZ) is a recombinant human IL-6 monoclonal antibody, which specifically binds to membrane-bound IL-6 receptors (IL-6R), thereby blocking IL-6 signaling and its mediated inflammatory response. Along with basic antivirus treatment, TCZ was administered to 20 patients (400 mg once a day, intravenously) and within a few days, the fever returned to normal and other symptoms improved markedly. Seventy percentage showed improvement in oxygenation and the opacity lung injury on CT scans absorbed in 90.5% of the patients. In addition, the percentage of peripheral lymphocytes returned to normal in 52.6% of the patients. Their data suggest that TCZ may be an effective alternative for the treatment of critically ill patients with COVID-19 (9, 53, 54).

Previous reports have shown that the administration of corticoid therapy to patients with immunological disorders has improved their health status (55). The use of 6 mg/day of

dexamethasone reduced the mortality rate of patients when compared to those without corticoid treatment. In addition, a reduction in mortality was observed in one third of the patients who received invasive mechanical ventilation and in one fifth who received oxygen without invasive mechanical ventilation. However, the treatment did not reduce mortality in those who did not receive respiratory support. These findings reveal that the use of dexamethasone (6 mg/day) for up to 10 days reduced mortality by 28 days in patients with COVID-19 who received invasive mechanical ventilation (55).

A randomized clinical trial compared the mortality rate between a group of patients treated with dexamethasone (2,104 patients) and another group treated with the usual care (4,321 patients). Overall, 482 patients (22.9%) in the dexamethasone group and 1,110 (25.7%) in the usual care group died 28 days after randomization. The proportional and absolute differences between groups in respect to mortality rate varied considerably according to the level of respiratory support at the time of randomization. In the dexamethasone group, the incidence of death was lower than that of the usual care group among patients with invasive mechanical ventilation (29.3 vs. 41.4%) and among those receiving oxygen without invasive mechanical ventilation (23.3 vs. 26.2%), but not among those who did not receive respiratory support at randomization (17.8 vs. 14.0%) (55).

Corticosteroid administration for a short period of time in patients with COVID-19 has improved the prognosis of the disease, resulting in decreased mortality and intubation (56). Such findings were corroborated by Selvaraj et al. (57) who reported that the short-term use of systemic corticosteroids by hospitalized patients with SARS-CoV-2 with hypoxic respiratory failure was well-tolerated and that the majority of the patients had an improvement in their prognosis. In addition, other studies have shown that short-term use of corticosteroids alleviates the severity of inflammation and reduces the mortality rate (38, 58). These findings support the use of corticosteroids during the ideal window of time to help alleviate the severity of inflammation and ultimately prevent the phase of severe hyperinflammation. However, a thorough clinical evaluation of each patient is mandatory before initiating corticosteroid therapy. Glucocorticoid treatment in patients with initial C-reactive protein (CRP) above 20 mg/dL has been associated with a significantly reduced risk of mortality and a decreased need for mechanical ventilation, whereas glucocorticoid treatment in patients with CRP below 10 mg/dL has been associated with significantly increased risk of mortality and higher need for mechanical ventilation (59).

There are several reports on the administration of anti-inflammatory drugs for the treatment of COVID-19. Some studies demonstrate the benefits of using corticosteroids in low doses for a short period of time during stage III of COVID-19. The use of corticosteroids needs to be well-evaluated, as other drugs can treat hyperthermia and inflammatory processes more selectively. In general, many studies indicate that there is no single reason to expect patients with SARS-CoV-2 infection to benefit from corticosteroids use but are otherwise more likely to be harmed by this treatment. Based on the studies analyzed in this review, corticosteroid administration to patients with COVID-19 may more likely inhibit immune responses and increase the rate of bacterial infection, which would probably prolong hospitalization and increase mortality rate.

Antivirals and Their Use in the Context of SARS-CoV-2

In order to find specific antiviral treatment for the new coronavirus, tests with broad spectrum drugs have been carried out. In addition, a screening of existing chemicals capable of affecting the transcription mechanisms from different cell perspectives has identified some promising drug candidates. Finally, the development of new specific antiretroviral drugs based on the genomic characteristics and viral behavior of SARS-CoV-2 has also been considered (60).

The use of interferon-alpha (IFN-α) at 5.000 U twice a day (bis in die—BID) and lopinavir/ritonavir (400/100 mg twice a day through oral route) has been recommended by Chinese guidelines. IFN-α is a broad-spectrum antiviral while lopinavir is a protease inhibitor and ritonavir enhance lopinavir activity by increasing its half-life. The use of lopinavir/ritonavir showed to be more effective than ribavirin alone as the patients treated with the former association showed lower rates of progression to acute respiratory distress syndrome (ARDS) and mortality (20, 61).

Nucleoside Analogs

Favipiravir and ribavirin are the main nucleoside analogs, whose mechanism of antiviral activity involves the induction of lethal mutagenesis of some viruses, such as influenza. In fact, the use of favipiravir associated with oseltamivir in the treatment of severe influenza showed to be more effective than oseltamivir alone (21). However, there is an exonuclease expression in non-structural protein 14 (nsp14-ExoN) in the coronavirus families, which may point to SARS-CoV-2 being resistant to this category of antivirals, corroborating studies in which ribavirin and favipiravir showed relatively poor activity against coronavirus (62).

Remdesivir is another nucleoside analog that has shown previous activity against Ebola and Nipah virus infection, whose mechanism of action involves the inhibition of RNA-dependent RNA polymerase (RdRp), therefore, it can inhibit the replication of coronaviruses. In fact, remdesivir has demonstrated activity against both SARS and MERS, whose antiviral effect might potentially be extrapolated to SARS-CoV-2 (**Figure 5**) (63).

A molecular docking study was carried out involving ribavirin, remdesivir, sofosbuvir, galidesivir and tenofovir against SARS-CoV-2 RdRp. These are anti-polymerase drugs that are currently on the market and have been previously approved for use as antivirus. All five drugs were able to tightly bind to the new coronavirus strain RdRp and therefore, are considered promising candidates to treat COVID-19 (64).

Remdesivir has shown a promising antiviral effect against COVID-19 in mild to moderate clinical situations. In an experimental animal study, the rodent groups infected with the Middle East Respiratory Syndrome (MERS)-CoV that received remdesivir showed an effective reduction in viral load when compared to the control group. An improvement in the damage to the lung parenchyma was observed, which promoted better

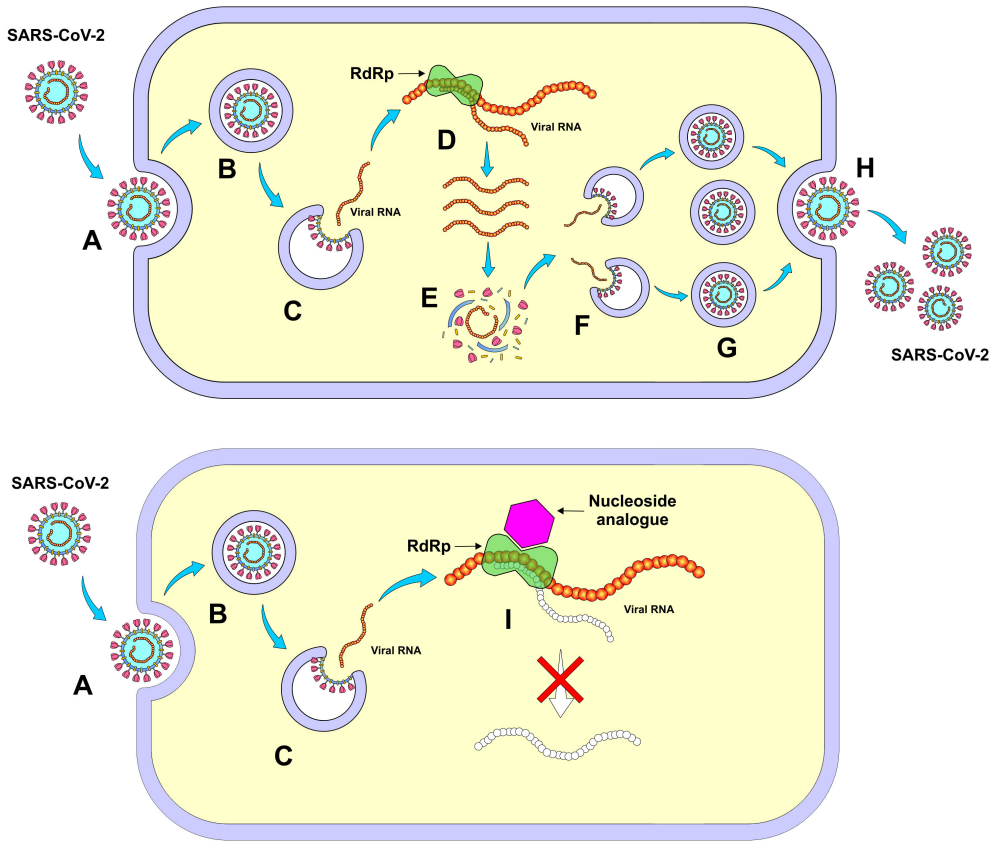


FIGURE 5 | Mechanism of action of nucleoside analogs (Remdesivir) through inhibition of RNA-dependent RNA polymerase (RdRp). **(A)** Entry of SARS-CoV-2 into the cell, **(B)** Formation of the endosome, **(C)** Release of viral RNA in the cell's cytoplasm, **(D)** Viral self-replication by RdRp, **(E)** Synthesis of virus structural proteins, **(F)** Incorporation of RNA viral, **(G)** New viral units, **(H)** Release of new viruses, and **(I)** Inhibition of viral self-replication by RdRp. *Figure source: Authors' own drawing*.

local tissue recovery compared to the group treated with lopinavir and ritonavir in combination with IFN-β (64).

In a randomized phase 3 study, whose inclusion criteria included SARS-CoV-2 infection and O_2 saturation equal to or <94% with pneumonia, 200 mg of remdesivir was administered intravenously on day 1 and 100 mg on subsequent 5 or 10 days. Three hundred ninety-seven patients were used in this study, 200 under a 5-days regimen and 197 under a 10-days regimen. In general, patients who underwent 10-days treatment had a significant clinical worsening in relation to the 5-days group. Most common adverse reactions were nausea (9% of reports), worsening of respiratory failure (8%), elevation of alanine aminotransferase level (7%), as well as constipation (7%) (22).

Sixty four percentage of the patients from the 5-days regimen group had recovered, compared to 54% from the 10-days group. However, this study has some limitations such as the absence of a randomized and placebo-controlled trial as well as the lack of analysis of viral load for SARS-CoV-2 during and after treatment. In addition, this study showed no significant difference in terms of efficacy between 5 and 10 days of treatment with intravenous remdesivir in patients with severe COVID-19 who did not need mechanical ventilation. For patients with the need for mechanical ventilation, the 10-day regimen was more effective, although it needs more in-depth studies among risk groups and immunocompromised patients in order to identify the effectiveness of the shorter-duration treatment (22).

An investigator-initiated, randomized, placebo-controlled, multicentered, double-blinded trial was conducted using intravenous remdesivir in 155 patients that were positive for SARS-CoV-2 and had chest imaging suggestive for pneumonia, oxygen saturation of 94% and FIO_2 ≤300 mmHg. Patients received intravenous remdesivir (200 mg/day on the first day and 100 mg/day on days 2–10), whereas the placebo group received the same infusion volume of a placebo solution for a total of 10 days. The results showed that the rate of clearance of the virus and mortality were not significantly changed in the group treated with intravenous remdesivir, however, an overall reduction of nearly 5 days in the median time of improvement of the clinical manifestations was observed. There was no significant reduction in the viral loads and in the duration of invasive mechanical ventilation. There were reports of adverse events in 66% of the patients in the remdesivir group and in 50% of those in the placebo group (23). It is important to emphasize the need for additional studies with larger samples, as well as strategies to

enhance the effectiveness of remdesivir by either using higher doses or associating with other antivirals/antibodies that could possibly neutralize SARS-CoV-2.

Neuraminidase Inhibitors

Chen et al. (24) studied the use of neuraminidase inhibitors such as oseltamivir (75mg twice a day through oral route) in 75 patients receiving treatment with non-specific antivirals such as ganciclovir (guanine nucleotide analog, 0.25 g twice a day intravenously) and the aforementioned association of lopinavir with ritonavir (500 mg twice a day, oral). Associations with antibiotics of different classes, as well as with corticosteroids were also investigated but with no description about the specific outcome in relation to the hospitalization period and death rate (24).

The use of neuraminidase inhibitors such as oseltamivir and zanamivir can influence influenza-like manifestations resulting in a decrease in the duration of symptoms. They can be used in situations of mild respiratory manifestations and therefore, it might be considered as a preventive measure among the strategies for flattening the curve and for preventing the collapse of health systems due to non-specific problems related to COVID-19 (65). On the other hand, since the new coronavirus does not synthesize neuraminidase (62), it seems likely to infer that this class of antivirus might not be effective for SARS-CoV-2.

Ivermectin

Ivermectin's antiviral activity has been proven *in vitro* against several viruses, including influenza, dengue, viral encephalitis and HIV. It acts by inhibiting the integrase protein and importin α/β1 (IMPα/β1) heterodimer which helps the former to be inserted into the nucleus during the interaction between HIV-1 and the human cell, resulting in interruption of viral replication (**Figure 6**). It is believed that the activity of IMPα/β1 on RNA viruses is what designates the broad spectrum of ivermectin (66). In addition, ivermectin stimulates GABA-gated chloride channels that ends up triggering a hyperpolarization process, resulting in paralysis of the infecting organism. Another proposed mechanism involves the immunomodulation of the host's response through the activation of neutrophils, with increased levels of C-reactive protein and IL-6 (67).

Another study investigated the hypothesis that IMPα/β1 influences the closure of the signal-dependent nucleocapsid protein of the SARS-CoV-2 nucleus-cytoplasmic, which impacts on the division of the host cell. Accessory protein ORF6 plays a role in antagonizing the activity of the transcription factor STAT1 by capturing IMPα/β1 in the membrane of the rough endoplasmic reticulum/Golgi membrane. This study was undertaken by infecting Vero/hSLAM cells with SARS-CoV-2 from the isolated strain Australia/VIC01/2020, followed by the addition of 5 μM of ivermectin. On days 0–3, supernatant cell materials were collected for RT-PCR analysis for SARS-CoV-2. As a result, a 93% reduction in the viral supernatant RNA (indicative of released virions) was found after 24 h. At 48 h, the reduction in viral RNA in the ivermectin-treated group increased to approximately 5,000 times compared to the control group, with no viral replication within 72 h (25).

Despite the broad antiviral spectrum of ivermectin found mostly *in vitro*, it is necessary to emphasize that clinical trials need to be conducted in order to better correlate the results of animal models to humans. It is worth noting that there was no evidence of reproducibility of the results found in infected rat models, which reinforces the FDA statement in April 2020 about the risks of self-medication with ivermectin against COVID-19. *In vitro* studies with promising results represent only the first stage of drug development. In addition, the studies that showed the efficacy of ivermectin on SARS-CoV-2 used doses within the microgram range. On the other hand, in humans, serum ivermectin levels for a safe therapeutic window should be around 20–80 ng/mL, which are considerably lower than those used in the *in vitro* experiments (68).

Nitazoxanide

Nitazoxanide has been considered as a therapeutic option for SARS-CoV-2. It is an antiparasitic agent approved by FDA for the treatment of Cryptosporidium and Giardia, in addition, it has shown a broad-spectrum antiviral activity against Noro and Rotavirus, as well as hepatitis B and C. Its mechanism of action is based on the increase in the sensitivity to cytoplasmic RNA and Interferon I pathways, which implies in regulating specific host cellular mechanisms dodged by the virus for its replication (69).

An ongoing clinical trial has shown the antiviral activity of nitazoxanide against 16 viruses including Influenza A subtypes (H1N1, H3N2, H3N2v, h3n8, h5n9, h7n1), Influenza B, respiratory syncytial viruses, dengue fever, yellow fever, Japanese encephalitis virus, rotavirus, HIV, SARS and MERS. A randomized, double blind study with 86 participants is currently being undertaken in Mexico where it compares the use of Hydroxychloroquine (200 mg, 12/12 h for 10 days) vs. Hydroxychloroquine + Nitazoxanide (200 + 500 mg, 12/12 h for 10 days). This latter association aims to decrease the hyperinflammatory process that leads to the evolution of the respiratory condition (26).

Azithromycin

Epithelial tight junction functions as a barrier that impedes the entrance of pathogenic microorganisms to several organs and tissues, such as lungs. Coronaviruses seem to disrupt the epithelial tight junctions by downregulating proteins involved in the maintenance of their integrity, which increases the potential for SARS-CoV-2 invasion and penetration. Conditions known to increase risk for COVID-19 complications include advanced age, diabetes mellitus, smoking and chronic lung disease. All these conditions are associated with higher predisposition to dysfunction of tight junctions. Moreover, SARS-CoV-2 infected patients have been reported to have increased levels of IL-6, TNF-alpha and interferon-gamma, all of which have shown to impair tight junctional function in several epithelial cell lines (**Figure 7**) (70).

It is believed that azithromycin can limit the growth of pathogens that disrupt intercellular tight junctions. This drug

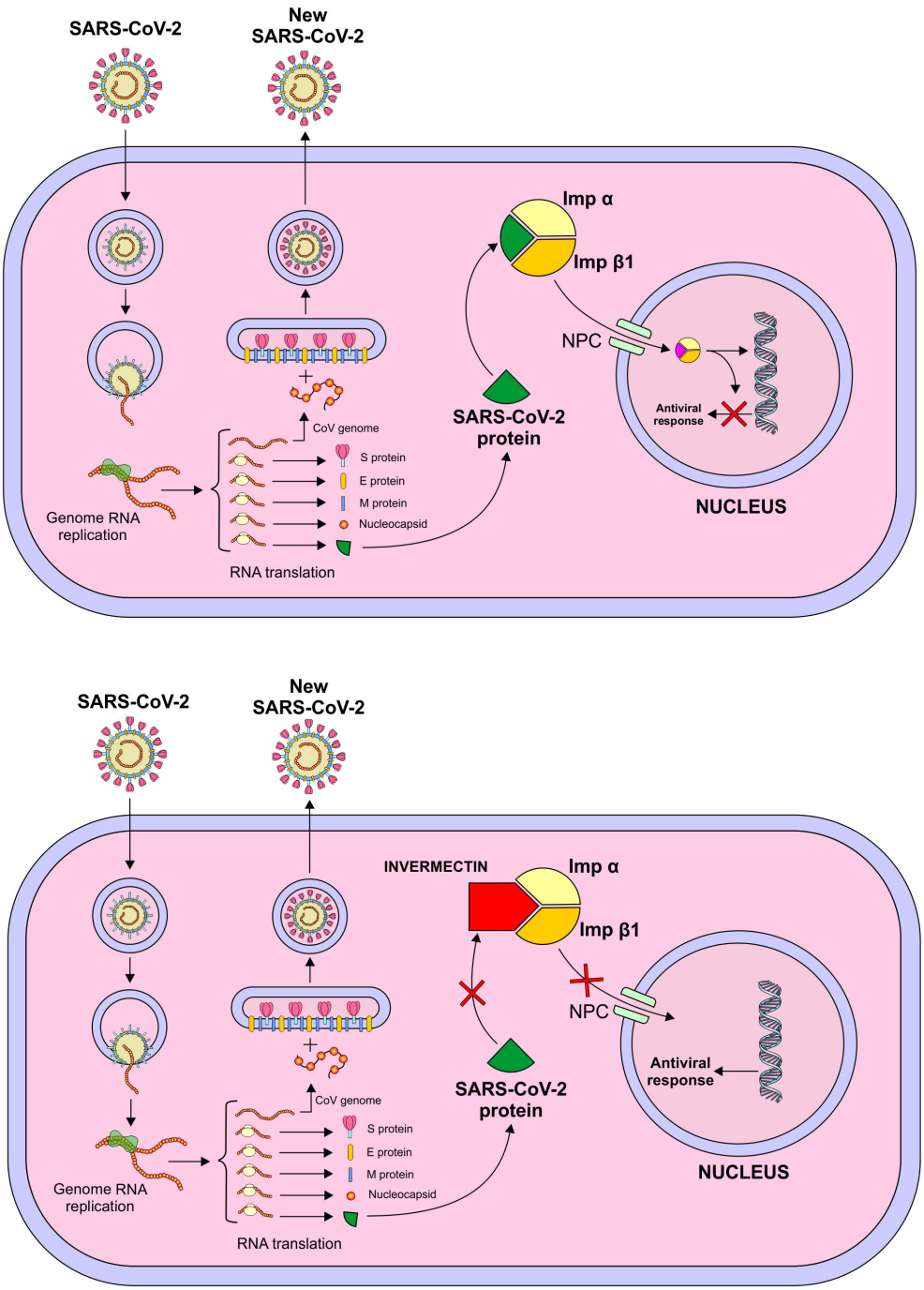


FIGURE 6 | Mechanism of action of Ivermectin – Inhibition of integrase protein and importin α/β1 (IMPα/β1) heterodimer which helps the former to be inserted into the nucleus during the interaction between the virus and the human cell, resulting in interruption of viral replication. *Figure source: Authors' own drawing*.

alters the processing and location of natural sealant molecules, which seems to exhibit a sealing effect on respiratory tight junctions (71). In addition, azithromycin has long been used to prevent respiratory tract infections caused by virus (72). It has shown to be effective against some viruses such as influenza (73), Zika (74), and Ebola (75).

Some preliminary data is available to support the use of azithromycin with hydroxychloroquine for treatment of patients with COVID-19, even though the success of its use may be limited to patients at the peak of COVID-19 symptoms and in potential respiratory collapse. A clinical study conducted in France showed that the association of hydroxychloroquine (600

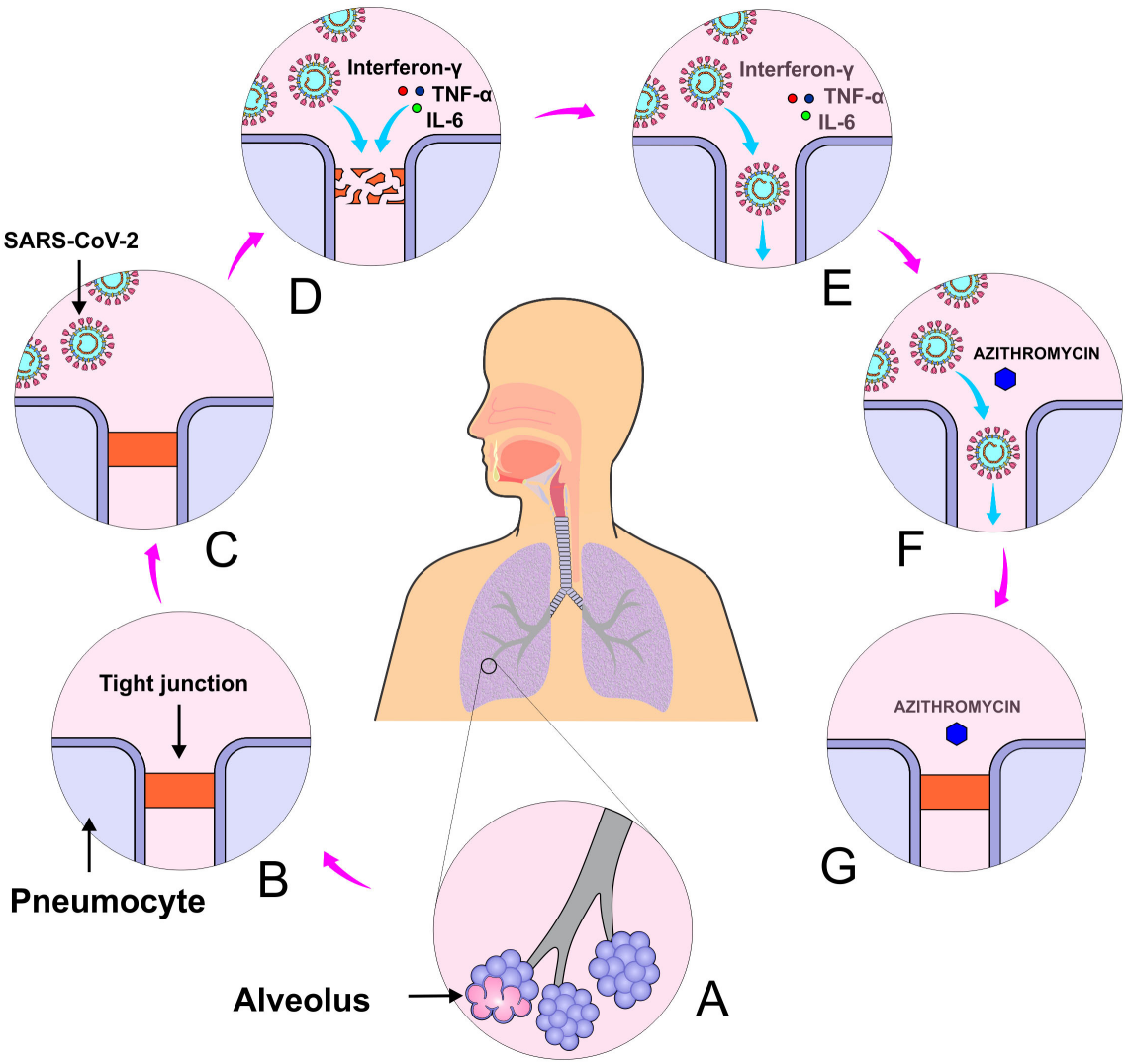


FIGURE 7 | Azithromycin activity in maintaining the integrity of tight junctions. **(A)** Alveoli, **(B)** Tight junction, **(C)** Lung infection by SARS-CoV-2, **(D)** Tight junction disruption caused by virus interleukin 6, tumor necrosis factor and interferon α, **(E)** SARS-CoV-2 entrance, **(F)** Administration of azithromycin, and **(G)** Restoration of tight junctions.

mg/day for 10 days) with azithromycin (500 mg/day on the first day followed by 250 mg/day on the next 4 days) was advantageous as 100% of the individuals treated with this association were virologically cured compared with 57.1% of those treated with only hydroxychloroquine (12). Despite its small sample size (26 patients in the treated group and 16 in the control group), this study opens the possibility of a synergistic effect of the combination of azithromycin with hydroxychloroquine.

Gabriels et al. (76) advert that the combination of hydroxychloroquine and azithromycin can prolong the QT interval and therefore may increase the arrhythmogenic risk of the patients submitted to such treatment. The authors raised the importance of cardiac rhythm monitoring in SARS-CoV-2 positive patients under hydroxychloroquine + azithromycin treatment, especially those with prior history of atrial fibrillation.

Although azithromycin has shown potential activity in maintaining the integrity of pulmonary epithelial tight junctions, a question arises whether it could aid in ameliorating pulmonary compromise in those patients in which SARS-CoV-2 has already penetrated the respiratory epithelium with the patient exhibiting pulmonary complications. Therefore, it seems likely to infer that the sealing activity of azithromycin on respiratory epithelial tight junctions might be useful for prophylaxis of COVID-19. In addition, its widespread use during this pandemic wave might increase the risk of antibiotic resistance and therefore, the pay-off must be worthwhile.

The use of azythromycin in combination with hydroxychloroquine has been investigated. Retrospective study involving 2,541 hospitalized patients with a mean age of 64 revealed a significant reduction in mortality among those

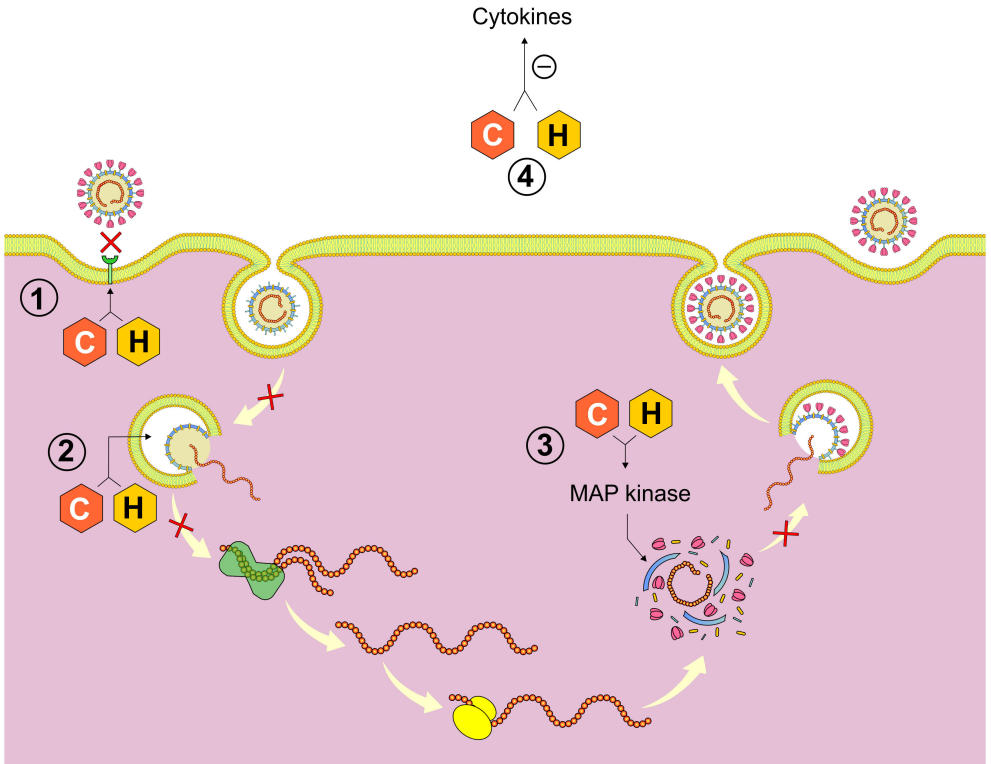


FIGURE 8 | Mechanisms of action of chloroquine (C) and hydroxychloroquine (H). (1) Inhibition of host glycosylation receptor and quinone reductase 2 responsible for the formation of the sialic acid necessary for the incorporation of the virus into the host cell, (2) Alteration of the endosome pH and inhibition of cathepsins responsible for the extrusion of the viral RNA of the endosome, (3) MAP kinase inhibition interfering with the proteolytic processing of protein M, and (4) Immunomodulatory effect resulting in inhibition of the synthesis of cytokine. *Figure source: Authors' own drawing*.

who received the combination of hydroxychloroquine and azithromycin. However, the authors emphasize that prospective studies are essential to confirm the impact of such association in comparison with the use of hydroxychloroquine alone (77).

Chloroquine and Hydroxychloroquine

Chloroquine and hydroxychloroquine are drugs derived from 4-aminoquinolines that have been reported to inhibit SARS-CoV2 by blocking viral entry through the inhibition of host receptor glycosylation, as well as through proteolytic processing and endosomal acidification. In addition, immunomodulatory effects have been attributed to these drugs through inhibition of cytokine production, autophagy and lysosomal activity in host cells (**Figure 8**). Hydroxychloroquine seems to have a greater antiviral activity (EC50 = 0.72 μM) besides having more tolerable safety profile, which makes it the preferred drug to treat malaria and autoimmune diseases such as rheumatoid arthritis, lupus erythematosus and dermatological conditions caused or aggravated by sunlight (78).

Based on preliminary results (79) carried out since January 2020, Chinese, South Korean, American and Brazilian health authorities have recommended the use of chloroquine and hydroxychloroquine for the treatment of SARS-CoV-2. Some clinicians consider their use reasonable in hospitalized patients with severe illness due to SARS-CoV-2 who are not eligible for hydroxychloroquine and chloroquine trials (80, 81).

In a randomized double-masked, phase IIb clinical trial (ChlorCovid-19 Study), with 81 adults who were hospitalized with SARS-CoV-2, preliminary findings suggest that the highest dose of chloroquine (600 mg, 2x/day, for 10 days, total dose of 12 g) should not be recommended for critically ill patients with COVID-19 due to security risk. These findings prematurely interrupted the recruitment of patients for this study (17, 82, 83).

The ideal dosage is uncertain; the FDA suggests hydroxychloroquine 800 mg on the first day followed by 400 mg daily and chloroquine 1 g on day 1 followed by 500 mg daily. Duration of treatment varies from four to seven days depending on the clinical response. Other hydroxychloroquine regimens includes: 1–400 mg twice a day on day 1 followed by 400 mg once a day for 5 days; 2–400 mg twice a day on day 1 and 200 mg twice for 4 days and 3–600 mg twice daily on day 1 and 400 mg daily for four days (84, 85).

The clinical data available for the use of hydroxychloroquine and chloroquine against COVID-19 is limited and its effectiveness is so far unknown. In the United States, the FDA has authorized the emergency use of these drugs in adolescents and adults hospitalized by COVID-19 when participation in clinical trials is not feasible. However, if these agents are used outside a clinical trial, the possibility of drug toxicity (including

prolongation of the QTc interval, drug-induced *torsades de pointes*—a form of polymorphic ventricular tachycardia, as well as cardiomyopathy and retinal toxicity) are more likely to occur. Gastrointestinal responses, such as vomiting and diarrhea, are the most common adverse effects of these two drugs. Previous reports have shown that patients with long-term exposure to chloroquine suffer from severe side effects, such as retinopathy, circular defects (or bull eye maculopathy) and diametric defects in the retina. In addition, drug interactions should be considered before use, especially in individuals who may be at risk. Thus, more susceptible patients should be monitored closely for side effects during chloroquine/hydroxychloroquine use. In fact, the American College of Cardiology suggests QTc monitoring in those patients at risk under the use of these drugs (80, 86–89).

Recent retrospective studies show a reduction in mortality among COVID-19 hospitalized patients who received the combination of hydroxychloroquine and azithromycin compared to those who underwent other treatments (13). However, a recently published multicenter randomized controlled trial with 504 patients with mild-moderate COVID-19 showed that the use of hydroxychloroquine at a dose of 400 mg twice daily alone or with azithromycin at a dose of 500 mg once daily for 7 days did not improve clinical status at 15 days when compared with standard care (16).

Two recently published randomized controlled trials investigated the use of hydroxychloroquine in reducing the severity of symptoms in adults with mild COVID-19. In the first study (15), 491 adults, of which 341 had laboratory-confirmed infection, were randomized into 2 groups that received either 800 mg of hydroxychloroquine on the first day and 600 mg/day on the next five days or equivalent doses of placebo. In the second study, 136 adults were randomly treated with 800 mg of hydroxychloroquine on the first day and 400 mg/day on the following six days and 157 did not receive this treatment (14). Both studies showed that hydroxychloroquine was not effective in reducing the severity of symptoms and that the rate of hospitalization was not significantly lower among those not treated with this drug.

Based on these findings, randomized clinical trials have not proven the efficacy of hydroxychloroquine alone or combined with azithromycin in reducing the duration and severity of symptoms in adults with mild-moderate COVID-19.

Convalescent Plasma

Plasma therapy consists in administering to patients with infectious diseases and severe conditions the plasma itself, or fractionated antibodies, along with other immunoglobulins obtained from donors who are in the stage of convalescence of the infection or have been cured. This therapy has been used since the Spanish flu pandemic between 1917 and 1918, as well as in other pandemics due to infectious diseases, being the last record of studies in the Ebola epidemic between 2013 and 2015 (5, 90).

A study carried out with 69 Ebola infected patients (44 receiving plasma therapy) between 2014 and 2015 revealed a significant reduction in viral load after 24 h of treatment, even though no significant reduction in mortality was observed. However, the authors highlighted the promising effects of this therapy, although the study sample was small and randomized clinical trials with a larger sample is needed in order to confirm its efficacy (91).

During the first epidemic of SARS caused by coronavirus between 2002 and 2003, several studies involving plasma therapy were published, and the majority revealed a significant reduction in viral load and improvement of symptoms among treated patients (5). Subsequently, a meta-analysis involving 32 studies with patients infected with influenza and SARS coronavirus showed that convalescent plasma therapy was safe and may have reduced the mortality of these patients even though the study was biased and the quality of the evidence was low. In fact, the authors recommended conducting clinical trials with an appropriate methodology to better assess the effectiveness of this therapy (92).

To date, two studies have been published on the use of convalescent plasma in patients infected with the new coronavirus. Shen et al. evaluated a series of 5 cases of SARS-CoV-2 with severe symptoms characterized by pneumonia with rapid progression, PaO2/FiO2 $\leq$ 300 mmHg, under mechanical ventilation and with high viral load despite treatment with antivirals. All patients were treated with transfusions of convalescent plasma and it was demonstrated that body temperature was normalized in 4 patients after 3 days, whereas a decrease in the Sequential Organ Failure Assessment (SOFA) score, negative viral load and increased PaO2/FiO2 were observed after 12 days of treatment. However, the authors recommended more robust clinical trials in order to confirm these findings (93).

In the second study, 10 critically ill patients infected with the new coronavirus received a transfusion of 200 mL of convalescent plasma donated by people who had recently recovered from infection with antibody titers above 1:640. The authors stated that there was a significant improvement in oxygen saturation after 3 days, a decrease in C-reactive protein, varied absorption of lung lesions in radiological exams after 7 days and undetectable viral load in 7 patients without any serious side effect. However, they highlighted the need for randomized clinical trials with the purpose of defining the ideal dose and the best time for administration of convalescent plasma (19).

Finally, Roback and Garner stated in a recently published editorial that the use of convalescent plasma is not new as it had been tested in the pandemics of avian influenza (H5N1), influenza in 2009 (H1N1) and Ebola. The study by Cheng et al., who in 2003 tested the therapy in Honk Kong patients with SARS coronavirus, found that among the 80 patients who received plasma transfusions, the mortality rate was significantly lower than the 299 who did not receive the treatment. However, considering its use in cases of SARS-CoV-2, the authors warned that the administration of convalescent plasma has not yet been evaluated in randomized clinical trials. Therefore, it cannot be guaranteed that the improvement in the symptoms was due only to this intervention as the patients received additional drugs, such as corticosteroids and antivirals, that may have influenced the improvement of the condition. On the other hand, they agreed that the study published by Shen et al. provides sufficient evidence for large clinical trials involving the administration

of convalescent plasma to critically ill patients with COVID-19 (19, 44, 93, 94).

The use of convalescent plasma associated with anticoagulants has been considered for patients with severe or life-threatening COVID-19 symptoms. However, a systematic review with 8 studies (no randomized clinical trial) and with 32 patients concluded that due to the high risk of bias and the low quality of evidence there is no certainty of the effectiveness and safety of convalescent plasma for hospitalized patients (95).

A randomized clinical trial that investigated the use of convalescent plasma in comparison with a standard treatment in 103 critically ill hospitalized patients showed no significant difference in the meantime for clinical improvement considering 28 days of follow-up (96). In this study, plasma was collected from adult donors aged between 18 and 55 with two negative PCRs before hospital discharge, who were asymptomatic and had left the hospital for more than 2 weeks. The authors pointed out that as the trial was terminated early, this study may not have enough power to detect an important clinical difference.

Spyropoulos et al. based on retrospective studies with hospitalized patients receiving anticoagulants, recommended the use of these drugs especially in those with high D-dimer (97). Likewise, in an article authored by an international collaboration of clinicians and investigators, the use of anticoagulant therapy to severe or life-threatening COVID-19 patients is recommended (98). However, there is a consensus in all these studies that randomized clinical trials are necessary in order to prove the effectiveness of plasma and anticoagulants.

Vitamins

In supportive care, it is recommended the continuous assessment of nutritional status of all patients infected with SARS-CoV-2 in which those at nutritional risk should receive nutritional support as soon as possible. It is also emphasized that even patients with COVID-19 who are not at risk of malnutrition should maintain an adequate intake of proteins (1.5 g/day) and calories (25–30 kcal/day). In addition, some vitamins and oligoelements may have the potential to benefit infected patients due to their anti-inflammatory, antioxidant and anti-viral properties (99).

Virtual screening and other computational techniques have been used to discover drugs against SARS-CoV, dengue and Ebola viruses. Kandeel and Nazawi (100) used virtual screening to access the binding ability of 20 FDA approved molecules including a broad-spectrum antiviral (ribavirin), anti-hepatitis B (telbivudine) and two vitamins (vitamin B12 and nicotinamide) to a crystal structure of SARS-CoV-2 main protease. The evaluated parameters included the docking scores, ligand efficiency as well as lipophilic and hydrogen bonding interactions. The results showed that vitamin B12 and nicotinamide were ranked at the 4th and 6th position, respectively. Although the authors suggest that both vitamins have potential to be used for COVID-19 treatment in combination with other drugs, *in silico* modeling needs to be validated *in vitro*.

Previous reports have shown that vitamin C is a promising alternative to reduce the susceptibility of high-risk individuals to infection of the lower respiratory tract under certain conditions (101). Therefore, a moderate amount of vitamin C supplementation may be a way to prevent COVID-19. In addition, it has been shown that reduced levels of vitamin D and vitamin E in cattle can lead to bovine coronavirus infection (102). This suggests that adequate supplementation of vitamin D and vitamin E must be tested in humans in order to verify whether they may increase human resistance to SARS-CoV-2.

One of the most deleterious consequences of the prolonged indoor stay (lockdown) during this COVID-19 pandemic is the reduced levels of circulating vitamin D as a result of the insufficient sunlight exposure. Low levels of vitamin D has been associated with higher susceptibility to infections. The receptors for vitamin D are highly expressed by several immune cells, such as monocytes as well as T and B lymphocytes. Therefore, vitamin D deficiency is associated with significantly higher risk of respiratory viral infection, which means that increased vitamin D intake must be considered as an additional prophylactic measure for SARS-CoV-2 respiratory infection. Adequate levels of vitamin D might be achieved by administering this vitamin as a dietary supplement or by consuming foods with relatively high content of vitamin D, such as fatty fish, cod liver oil and egg yolks (102).

The use of vitamin D is justified by the growing evidence that normal values of this vitamin in infected patients can enhance immunity against pathogen and improve immune recovery during treatment with antiretroviral (99, 102–107). In addition, previous studies have demonstrated the role of vitamin D in preventing asthma and in improving the severity of asthmatic symptoms (108). A systematic review and meta-analysis published in 2017 identified 25 eligible randomized, double-blind, placebo-controlled trials with a total of 11,321 participants, whose effect of vitamin D supplementation on the risk of acute respiratory tract infection was assessed. This study showed that vitamin D supplementation was able to significantly reduce the risk of acute respiratory tract infection in 100% of the participants, especially those with considerably low 25-hydroxyvitamin D levels. It is interesting to note that such protective effect was more pronounced in individuals who received daily doses of vitamin D instead of large boluses. This latter procedure has been associated with reduced efficacy of vitamin D and increased risk of adverse outcomes (109).

In vitro studies have shown that vitamin D actively participates in the respiratory homeostasis by increasing the expression of antimicrobial peptides and by affecting the replication of respiratory viruses. In addition, vitamin D preserves tight junctions, eradicates enveloped viruses by inducing cathelicidin and defensins and decreases the proinflammatory cytokines by the innate immune system, which prevents the cytokine storm that leads to pneumonia (**Figure 9**) (110). In fact, The British Medical Journal has recently published an editorial where many researchers included vitamin D deficiency as one of the putative risk factors for the novel coronavirus infection (111). Regarding the adequate dose of vitamin D, it depends on the severity of the hypovitaminosis D. It is recommended that the levels of circulating 25-hydroxyvitamin D should fall in the 40–60 ng/mL range for optimum protection against acute viral infections (112).

Patients with a deficit of 25-hydroxyvitamin D [25 (OH) D] should promptly have vitamin D supplied according to the results

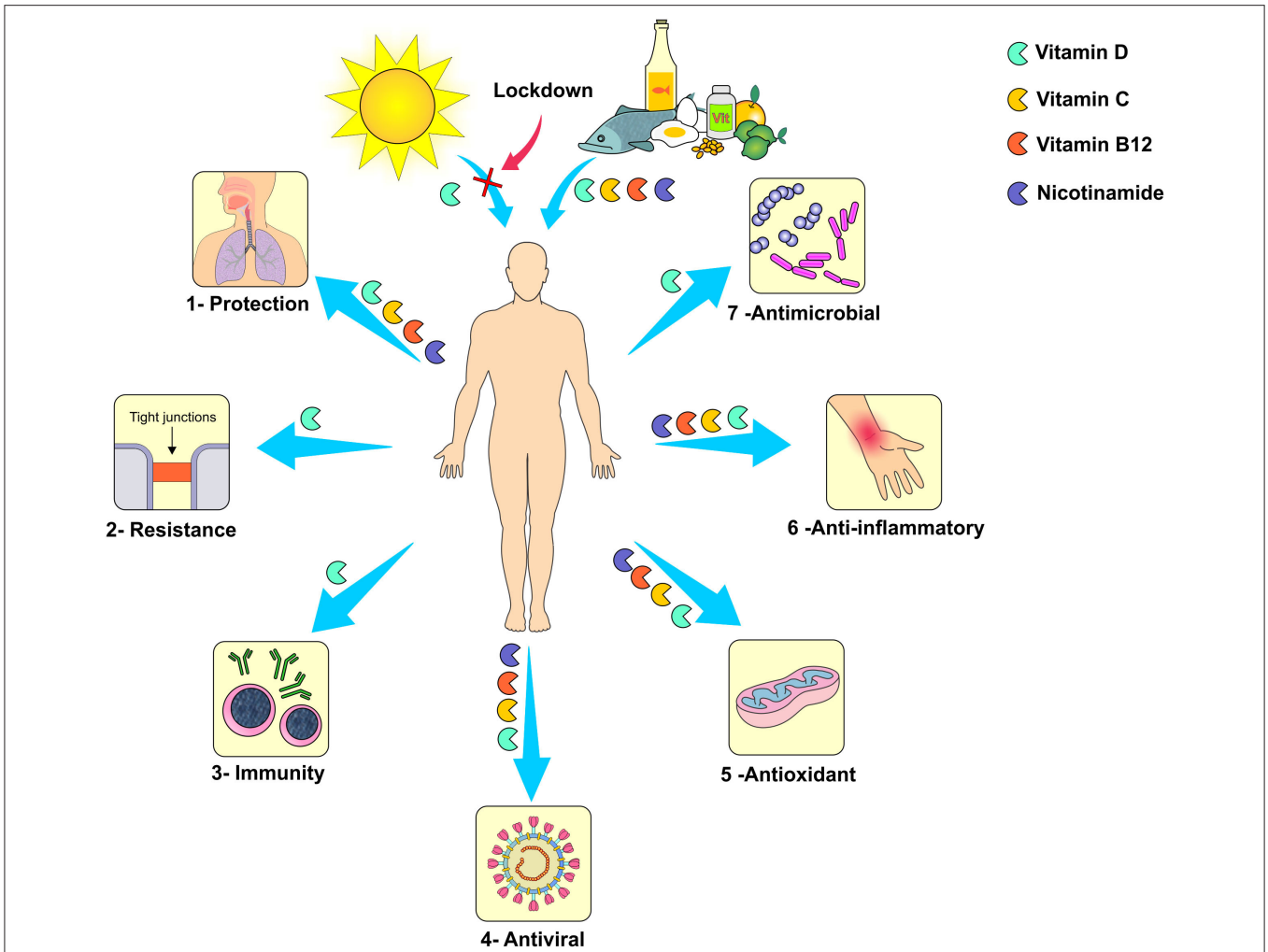


FIGURE 9 | Mechanism of action of vitamins: 1- Reduction of susceptibility to viral infections of the respiratory tract, 2- Maintenance of the integrity of intercellular tight junctions resulting in increased resistance to microorganisms penetration, 3- Improvement of immunity, 4- Direct antiviral activity, 5- Antioxidant activity, 6- Anti-inflammatory activity, and 7- Increased production of antimicrobial peptides. *Figure source: Authors' own drawing*.

of blood tests [50,000 IU/week when levels of 25 (OH) D <20 ng mL; 25,000 IU/week when levels of 25 (OH) D are between 20 and 30 ng/mL] (13, 15, 16, 84, 89). On the other hand, a recent review study stated that for people at risk for influenza and/or COVID-19 a daily dose of 10,000 IU of vitamin D for a few weeks should be considered and once the levels of 25(OH)D increases, the daily dose of vitamin D should decrease to 5,000 IU (27).

Although several studies have demonstrated the role of vitamin D in the maintenance of immune homeostasis, a randomized controlled trial is still needed in order to confirm that adequate vitamin D intake can prevent respiratory tract viral infections such as that caused by SARS-CoV-2. In addition, it is worth to point out that vitamin D supplementation should take place under proper medical supervision as hypervitaminosis D can result in irreversible calcification of soft tissues and although rare, it can be life-threatening.

Zinc

Zinc is an essential trace element that plays an important role in direct antiviral and immune responses. Such evidence can be confirmed by the higher risk of viral infections (Herpesviridae, HIV and Hepatitis C) in individuals with zinc deficiency. *In vitro* demonstration of the multiple mechanisms of antiviral actions of zinc has led to the indication of its supplementation as a preventive or therapeutic strategy to control viral infections (113–115).

An *in vitro* study demonstrated that the intracellular increase in Zn+2 associated with its ionophore pyrithione at concentrations of 2/2 μM, was able to inhibit viral replication of SARS-CoV and equine arteritis virus in cell cultures. According to the authors, the antiviral activity of Zn+2 is attributed to the inhibition of the RNA-dependent RNA polymerase (RdRp) responsible for the transcription of the viral genome (**Figure 10**) (116). Additionally, Read et al. pointed out that zinc has other

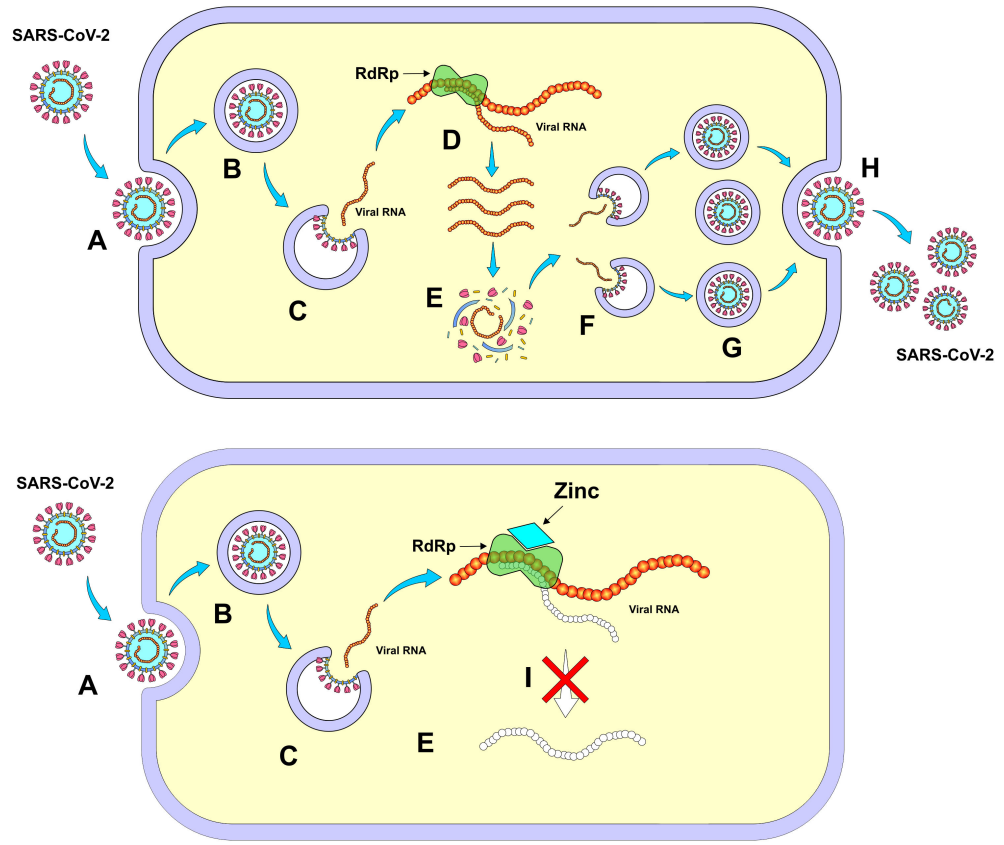


FIGURE 10 | Mechanism of action of zinc – Inhibition of RNA-dependent RNA polymerase (RdRp). **(A)** Entry of SARS-CoV-2 into the cell, **(B)** Formation of endosome, **(C)** Release of viral RNA in the cell cytoplasm, **(D)** Viral self-replication by RdRp, **(E)** Synthesis of virus structural proteins, **(F)** Incorporation of RNA viral, **(G)** New viral units, **(H)** Release of new viruses, and **(I)** Inhibition of viral self-replication by RdRp. *Figure source: Authors' own drawing*.

properties such as a direct inhibitory activity on other viruses, as well as inhibition of the formation of the viral coating and processing of its structural components (115).

The use of 75 mg/day of zinc has been able to reduce the severity of the cases and the period of illness in patients with viral infections. It is recommended though that the use of zinc should be started within the first 24 h of the onset of the infection symptoms and that its daily administration should be maintained throughout the disease period (117). On the other hand, Zhang and Liu state in their systematic review that the association of zinc and pyrithione at low concentrations contributes to the reduction of SARS replication (SARS-CoV), therefore having a direct antiviral effect (118).

Finally, Xue et al. demonstrated a synergistic effect of chloroquine with zinc in terms of the cytotoxic effect on cancer cells, opening a new possibility of association between antimalarial and zinc for other conditions such as viral infections (119).

DISCUSSION

At the time of writing this review, no drug has proven to be fully effective against COVID-19, however, regulatory agencies from all over the world are cautious by only supporting the use of agents whose effectiveness has been proved under certain conditions and based on promising results from reliable studies.

Throughout this review, scientific evidence for multiple therapeutic combinations was discussed, in which the studies have shown greater efficacy in comparison to each treatment individually. For instance, the increase in the effectiveness of the treatment has been documented for the association of hydroxychloroquine and azithromycin (77) as well as for lopinavir and ritonavir (20, 51, 62). In addition, in hospitalized patients with severe acute respiratory distress syndrome, interventions are indicated to control coagulation disorders and exacerbated inflammatory response as well as to increase the immune response. Thus, in severe or life-threatening COVID-19 patients, most guidelines indicate the association of anticoagulants, corticosteroids, antibiotics and immunity mediators. Furthermore, it seems that the use of multi-therapy by associating different therapeutic agents that act through distinct mechanisms of action is a promising alternative to overcome this current health crisis until a fully effective drug or vaccine is discovered.

In some countries, the prophylaxis of COVID-19 for health professionals, elderly and infected contacts has been proposed

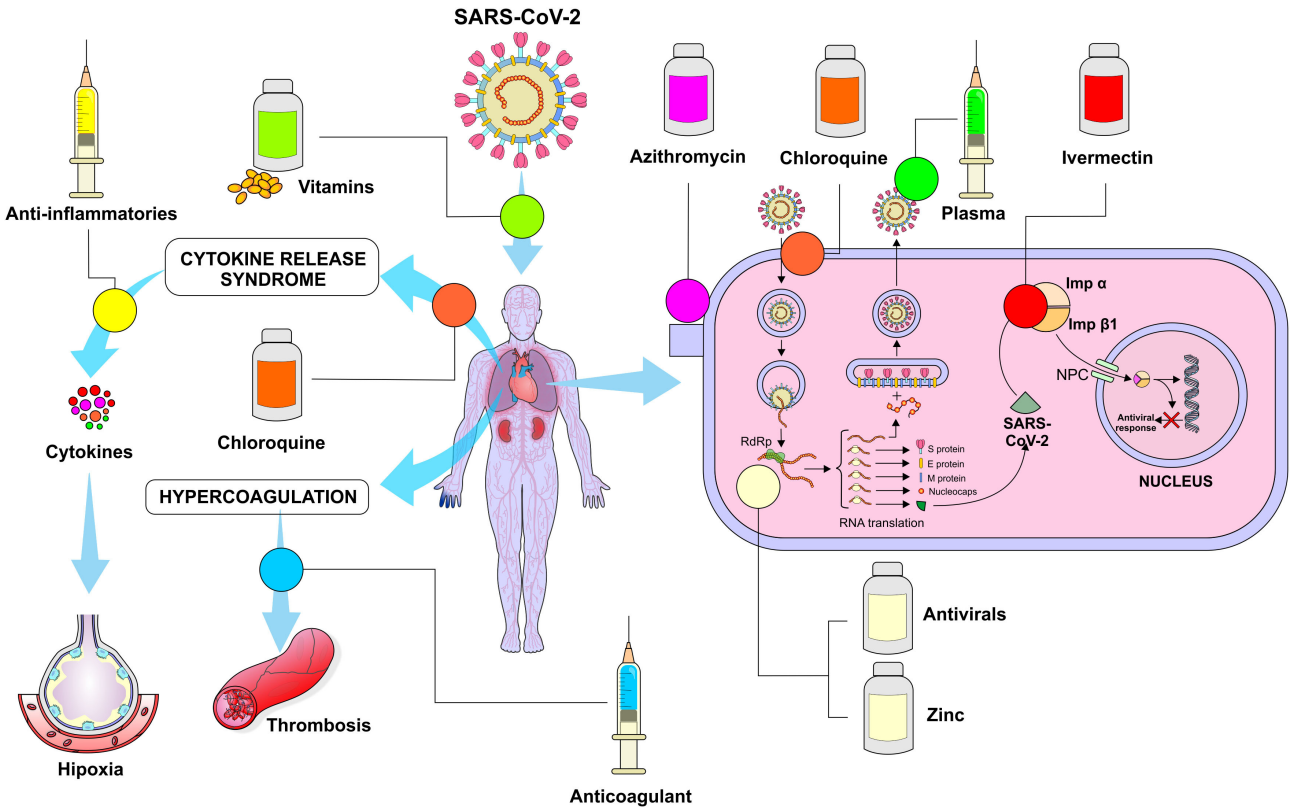


FIGURE 11 | Summary of the mechanisms of action of the drugs currently used in SARS-CoV-2 infection. Chloroquine – Interferes with ACE2 ligands and receptors, decreasing the penetration of the virus into the cell, in addition, it changes the pH of the endosome, making it difficult to release viral RNA in the cell's cytoplasm; Azithromycin - Reduces the number of microorganisms in the alveolus and maintains the integrity of tight junctions, reinforcing the virus barrier; Anti-inflammatory drugs - Reduce the inflammatory process by decreasing the release of cytokines, Ivermectin - Inhibits the integrase protein and importin α/β1 (IMPα/β1) heterodimer that promote the entry of viral proteins in the cell nucleus; Convalescent plasma - Immunoglobulins directly fight the virus; Anticoagulant drugs- Interact with antithrombin reducing the thrombotic process; Antivirals (Remdesivir) - Inhibit the RNA-dependent RNA polymerase (RdRp) preventing the self-replication of viral RNA; Zinc - inhibits RNA-dependent RNA polymerase (RdRp) preventing self-replication of viral RNA. *Figure source: Authors' own drawing*.

with drugs that have shown *in vitro* antiviral activity (67), which includes chloroquine, hydroxychloroquine and ivermectin. However, randomized clinical trials have not demonstrated a prophylactic effect with reduced hospitalization among adults with mild COVID-19 that have been treated with hydroxychloroquine. In addition, there are no randomized studies that prove the efficacy and safety of ivermectin prophylaxis against SARS-CoV-2. Therefore, the existing evidences do not support that the benefits of such prophylactic treatment outweigh the risks and we advise that all the risks must be clearly explained to patients who seek protection against SARS-CoV-2 by using these drugs.

Multiple treatment of COVID-19 has been adopted (**Figure 11**) from a responsible perspective, given the massive need to adopt important therapeutic measures in an increasingly intense relationship of disease severity and time. It is important to reinforce precautions regarding the side effects of some drugs and that in many cases only off-label and compassionate use are justified.

The development of more research to find a more specific drug to treat this disease has been ensured by the scientific community, preserving the bioethical principles of research involving human beings. We hope that as the several randomized clinical trials are being conducted worldwide, the drugs with the best efficacy and safety profile will soon be found.

AUTHOR CONTRIBUTIONS

AF wrote about antivirals, ivermectin, nitazoxanide, and organized the entire references. AV wrote about chloroquine and hydroxychloroquine. FG wrote about anti-inflammatory drugs, vitamins and helped with selecting the articles, and writing the conclusion section. FP wrote about zinc and drew all figures included in this manuscript. RC wrote about anticoagulants, convalescent plasma, organized the abstract, and methodology sections. EA wrote about azithromycin, vitamin D, and translated the entire text to English. All authors approved the final version of the manuscript before submission.

REFERENCES

1. Poon LC, Yang H, Kapur A, Melamed N, Dao B, Divakar H, et al. Global interim guidance on coronavirus disease 2019 (COVID-19) during pregnancy and puerperium from FIGO and allied partners: information for healthcare professionals. *Int J Gynaecol Obstet.* (2020) 149:273–86. doi: 10.1002/ijgo.13156
2. *WHO Director-General's Opening Remarks at the Media Briefing on COVID-19*. (2020). Available online at: https://www.who.int/dg/speeches/detail/who-director-general-s-opening-remarks-at-the-media-briefing-on-covid-19---15-june-2020 (accessed April 19, 2020).
3. *WHO Director-General's Opening Remarks at the Media Briefing on COVID-19*. (2020). Available online at: https://www.who.int/dg/speeches/detail/who-director-general-s-opening-remarks-at-the-media-briefing-on-covid-19---30-march-2020 (accessed April 19, 2020).
4. World Health Organization. *Coronavirus disease 2019 (COVID-19). Situation Report—163*. (2020). Available online at: https://www.who.int/emergencies/diseases/novel-coronavirus-2019/situation-reports (accessed July 2, 2020).
5. Syal K. COVID-19: herd immunity and convalescent plasma transfer therapy. *J Med Virol.* (2020) 92:1380–2. doi: 10.1002/jmv.25870
6. Li H, Zhou Y, Zhang M, Wang H, Zhao Q, Liu J. Updated approaches against SARS-CoV-2. *Antimicrob Agents Chemother.* (2020) 64:e00483-20. doi: 10.1128/AAC.00483-20
7. Bein B, Bachmann M, Huggett S, Wegermann P. [SARS CoV-2/COVID-19: evidence-based recommendation on diagnosis and therapy]. *Anasthesiol Intensivmed Notfallmed Schmerzther.* (2020) 55:257–65. doi: 10.1055/a-1146-8674
8. McCreary EK, Pogue JM. Coronavirus disease 2019 treatment: a review of early and emerging options. *Open forum Infect Dis.* (2020) 7:ofaa105. doi: 10.1093/ofid/ofaa105
9. Sanders JM, Monogue ML, Jodlowski TZ, Cutrell JB. Pharmacologic Treatments for coronavirus disease 2019 (COVID-19): a review. *JAMA.* (2020) 323:1824–6. doi: 10.1001/jama.2020.6019
10. Yao XH, Li TY, He ZC, Ping YF, Liu HW, Yu SC, et al. [A pathological report of three COVID-19 cases by minimally invasive autopsies]. *Zhonghua bing li xue za zhi.* (2020) 49:E009. doi: 10.3760/cma.j.cn112151-20200312-00193
11. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet.* (2020) 395:497–506. doi: 10.1016/S0140-6736(20)30183-5
12. Gautret P, Lagier J-C, Parola P, Hoang VT, Meddeb L, Mailhe M, et al. Hydroxychloroquine and azithromycin as a treatment of COVID-19: results of an open-label non-randomized clinical trial. *Int J Antimicrob Agents.* (2020) 56:105949. doi: 10.1016/j.ijantimicag.2020.105949
13. Lagier J-C, Million M, Gautret P, Colson P, Cortaredona S, Giraud-Gatineau A, et al. Outcomes of 3,737 COVID-19 patients treated with hydroxychloroquine/azithromycin and other regimens in Marseille, France: A retrospective analysis. *Travel Med Infect Dis.* (2020) 36:101791. doi: 10.1016/j.tmaid.2020.101791
14. Mitjà O, Corbacho-Monné M, Ubals M, Tebe C, Peñafiel J, Tobias A, et al. Hydroxychloroquine for Early treatment of adults with mild Covid-19: a randomized-controlled trial. *Clin Infect Dis.* (2020) 16:ciaa1009. doi: 10.1093/cid/ciaa1009
15. Skipper CP, Pastick KA, Engen NW, Bangdiwala AS, Abassi M, Lofgren SM, et al. Hydroxychloroquine in nonhospitalized adults with early COVID-19. *Ann Intern Med.* (2020) 16:M20-4207. doi: 10.7326/M20-4207
16. Cavalcanti AB, Zampieri FG, Rosa RG, Azevedo LCP, Veiga VC, Avezum A, et al. Hydroxychloroquine with or without azithromycin in mild-to-moderate Covid-19. *N Engl J Med.* (2020) NEJMoa2019014 1:12. doi: 10.1056/NEJMoa2019014
17. Borba MGS, Val FFA, Sampaio VS, Alexandre MAA, Melo GC, Brito M, et al. Effect of high vs low doses of chloroquine diphosphate as adjunctive therapy for patients hospitalized with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection: a randomized clinical trial. *JAMA Netw Open.* (2020) 3:e208857. doi: 10.1001/jamanetworkopen.2020.8857
18. Tang N, Bai H, Chen X, Gong J, Li D, Sun Z. Anticoagulant treatment is associated with decreased mortality in severe coronavirus disease 2019 patients with coagulopathy. *J Thromb Haemost.* (2020) 18:1094–9. doi: 10.1111/jth.14817
19. Duan K, Liu B, Li C, Zhang H, Yu T, Qu J, et al. Effectiveness of convalescent plasma therapy in severe COVID-19 patients. *Proc Natl Acad Sci USA.* (2020) 117:9490–6. doi: 10.1073/pnas.2007408117
20. HAN Archive - 00429 | Health Alert Network (HAN). (2020). Available online at: https://emergency.cdc.gov/han/2020/han00429.asp (accessed April 19, 2020).
21. Wang Y, Fan G, Salam A, Horby P, Hayden FG, Chen C, et al. Comparative effectiveness of combined favipiravir and oseltamivir therapy versus oseltamivir monotherapy in critically Ill patients with influenza virus infection. *J Infect Dis.* (2019) 221:1688–98. doi: 10.1093/infdis/jiz656
22. Goldman JD, Lye DCB, Hui DS, Marks KM, Bruno R, Montejano R, et al. Remdesivir for 5 or 10 days in patients with severe Covid-19. *N Engl J Med.* (2020) NEJMoa2015301:1–11. doi: 10.1056/NEJMoa2015301. [Epub ahead of print].
23. Wang Y, Zhang D, Du G, Du R, Zhao J, Jin Y, et al. Remdesivir in adults with severe COVID-19: a randomised, double-blind, placebo-controlled, multicentre trial. *Lancet.* (2020) 395:1569–78. doi: 10.1056/NEJMoa2007764
24. Chen N, Zhou M, Dong X, Qu J, Gong F, Han Y, et al. Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study. *Lancet.* (2020) 395:507–13. doi: 10.1016/S0140-6736(20)30211-7
25. Caly L, Druce JD, Catton MG, Jans DA, Wagstaff KM. The FDA-approved drug ivermectin inhibits the replication of SARS-CoV-2 in vitro. *Antiviral Res.* (2020) 178:104787. doi: 10.1016/j.antiviral.2020.104787
26. Rossignol JF. Nitazoxanide, a new drug candidate for the treatment of Middle East respiratory syndrome coronavirus. *J Infect Public Health.* (2016) 9:227–30. doi: 10.1016/j.jiph.2016.04.001
27. Grant WB, Lahore H, McDonnell SL, Baggerly CA, French CB, Aliano JL, et al. Evidence that vitamin D Supplementation could reduce risk of influenza and COVID-19 infections and deaths. (2020) 12:988. doi: 10.20944/preprints202003.0235.v2
28. Yin S, Huang M, Li D, Tang N. Difference of coagulation features between severe pneumonia induced by SARS-CoV2 and non-SARS-CoV2. *J Thromb Thrombolysis.* (2020) 3:1–4. doi: 10.1007/s11239-020-02105-8
29. Li H, Liu L, Zhang D, et al. SARS-CoV-2 and viral sepsis: observations and hypotheses. *Lancet.* (2020) 395:1517–20. doi: 10.1016/S0140-6736(20)30920-X
30. Ozolina A, Sarkele M, Sabelnikovs O, Skesters A, Jaunalksne I, Serova J, et al. Activation of coagulation and fibrinolysis in acute respiratory distress syndrome: A prospective pilot study. *Front Med.* (2016) 3:64. doi: 10.3389/fmed.2016.00064
31. Connors JM, Levy JH. COVID-19 and its implications for thrombosis and anticoagulation. *Blood.* (2020) 135:2033–40. doi: 10.1182/blood.2020006000
32. Li J, Li Y, Yang B, Wang H, Li L. Low-molecular-weight heparin treatment for acute lung injury/acute respiratory distress syndrome: a meta-analysis of randomized controlled trials. *Int J Clin Exp Med.* (2018) 11:414–22.
33. Seffer M-T, Cottam D, Forni LG, Kielstein JT. Heparin 2.0: a new approach to the infection crisis. *Blood Purif.* (2020) 1:1–7. doi: 10.1159/000508647
34. Thachil J, Tang N, Gando S, Falanga A, Cattaneo M, Levi M, et al. ISTH interim guidance on recognition and management of coagulopathy in COVID-19. *J Thromb Haemost.* (2020) 18:1023–26. doi: 10.1111/jth.14810
35. Gao Y, Li T, Han M, Li X, Wu D, Xu Y, et al. Diagnostic utility of clinical laboratory data determinations for patients with the severe COVID-19. *J Med Virol.* (2020) 92:791–6. doi: 10.1002/jmv.25770
36. Chen C, Zhang XR, Ju ZY, He WF. [Advances in the research of cytokine storm mechanism induced by corona virus disease 2019 and the corresponding immunotherapies]. *Zhonghua Shao Shang Za Zhi.* (2020) 36:E005. doi: 10.3760/cma.j.cn501120-20200224-00088
37. Qin C, Zhou L, Hu Z, Zhang S, Yang S, Tao Y, et al. Dysregulation of immune response in patients with COVID-19 in Wuhan, China. *Clin Infect Dis.* (2020) 71:762–8. doi: 10.1093/cid/ciaa248
38. Wu C, Chen X, Cai Y, Xia J, Zhou X, Xu S, et al. Risk factors associated with acute respiratory distress syndrome and death in patients with coronavirus disease 2019 pneumonia in Wuhan, China. *JAMA Intern Med.* (2020) 180:1–11. doi: 10.1001/jamainternmed.2020.0994

39. Conti P, Ronconi G, Caraffa A, Gallenga CE, Ross R, Frydas I, et al. Induction of pro-inflammatory cytokines (IL-1 and IL-6) and lung inflammation by Coronavirus-19 (COVI-19 or SARS-CoV-2): anti-inflammatory strategies. *J Biol Regul Homeost Agents.* (2020) 34:327–31. doi: 10.23812/CONTI-E
40. Ruan Q, Yang K, Wang W, Jiang L, Song J. Clinical predictors of mortality due to COVID-19 based on an analysis of data of 150 patients from Wuhan, China. *Intensive Care Med.* (2020) 46:846–8. doi: 10.1007/s00134-020-05991-x
41. Zhang W, Zhao Y, Zhang F, Wang Q, Li T, Liu Z, et al. The use of anti-inflammatory drugs in the treatment of people with severe coronavirus disease 2019 (COVID-19): The experience of clinical immunologists from China. *Clin Immunol.* (2020) 214:108393. doi: 10.1016/j.clim.2020.108393
42. Wang D, Hu B, Hu C, Zhu F, Liu X, Zhang J, et al. Clinical characteristics of 138 hospitalized patients with 2019 novel coronavirus-infected pneumonia in Wuhan, China. *JAMA - J Am Med Assoc.* (2020) 323:1061–9. doi: 10.1001/jama.2020.1585
43. Auyeung TW, Lee JSW, Lai WK, Choi CH, Lee HK, Lee JS, et al. The use of corticosteroid as treatment in SARS was associated with adverse outcomes: a retrospective cohort study. *J Infect.* (2005) 51:98–102. doi: 10.1016/j.jinf.2004.09.008
44. Jin YH, Cai L, Cheng ZS, Cheng H, Deng T, Fan YP, et al. A rapid advice guideline for the diagnosis and treatment of 2019 novel coronavirus (2019-nCoV) infected pneumonia (standard version). *Mil Med Res.* (2020) 7:4. doi: 10.1186/s40779-020-0233-6
45. Russell CD, Millar JE, Baillie JK. Clinical evidence does not support corticosteroid treatment for 2019-nCoV lung injury. *Lancet.* (2020) 395:473–5. doi: 10.1016/S0140-6736(20)30317-2
46. Arabi YM, Mandourah Y, Al-Hameed F, Sindi AA, Almekhlafi GA, Hussein MA, et al. Corticosteroid therapy for critically ill patients with middle east respiratory syndrome. *Am J Respir Crit Care Med.* (2018) 197:757–67. doi: 10.1164/rccm.201706-1172OC
47. Griffith JF, Antonio GE, Kumta SM, Hui DSC, Wong JKT, Joynt GM, et al. Osteonecrosis of hip and knee in patients with severe acute respiratory syndrome treated with steroids. *Radiology.* (2005) 235:168–75. doi: 10.1148/radiol.2351040100
48. Lee N, Allen Chan KC, Hui DS, Ng EKO, Wu A, Chiu RWK, et al. Effects of early corticosteroid treatment on plasma SARS-associated Coronavirus RNA concentrations in adult patients. *J Clin Virol.* (2004) 31:304–9. doi: 10.1016/j.jcv.2004.07.006
49. Ling Y, Xu S-B, Lin Y-X, Tian D, Zhu Z-Q, Dai F-H, et al. Persistence and clearance of viral RNA in 2019 novel coronavirus disease rehabilitation patients. *Chin Med J.* (2020) 133:1039–43 doi: 10.1097/CM9.0000000000000774
50. Siddiqi HK, Mehra MR. COVID-19 illness in native and immunosuppressed states: a clinical-therapeutic staging proposal. *J Hear Lung Transplant.* (2020) 39:405–7 doi: 10.1016/j.healun.2020.03.012
51. World Health Organization. *Clinical Management of Severe Acute Respiratory Infection When Novel Coronavirus (2019-nCoV) Infection is Suspected: Interim Guidance*. Geneva: World Health Organization. (2020). Available online at: https://extranet.who.int/iris/restricted/handle/10665/330893
52. Richardson P, Griffin I, Tucker C, Smith D, Oechsle O, Phelan A, et al. Baricitinib as potential treatment for 2019-nCoV acute respiratory disease. *Lancet.* (2020) 395:e30–1. doi: 10.1016/S0140-6736(20)30304-4
53. Lewis SR, Pritchard MW, Thomas CM, Smith AF. Pharmacological agents for adults with acute respiratory distress syndrome. *Cochrane Database Syst Rev.* (2019) 2019:CD004477. doi: 10.1002/14651858.CD004477.pub3
54. Shang L, Zhao J, Hu Y, Du R, Cao B. On the use of corticosteroids for 2019-nCoV pneumonia. *Lancet.* (2020) 395:683–4. doi: 10.1016/S0140-6736(20)30361-5
55. Horby P, Lim WS, Emberson J, Mafham M, Bell J, Linsell L, et al. Effect of dexamethasone in hospitalized patients with COVID-19: preliminary report. *medRxiv.* (2020) 1–11. doi: 10.1101/2020.06.22.20137273
56. Callejas Rubio JL, Luna del Castillo J de D, de la Hera Fernández J, Guirao Arrabal E, Colmenero Ruiz M, Ortego Centeno N. Effectiveness of corticoid pulses in patients with cytokine storm syndrome induced by SARS-CoV-2 infection. *Med Clin.* (2020) 155:159–61. doi: 10.1016/j.medcle.2020.07.002
57. Selvaraj V, Dapaah-Afriyie K, Finn A, Flanigan T. Short-term corticosteroids in SARS-CoV2 patients: hospitalists' perspective. *medRxiv.* (2020). doi: 10.1101/2020.06.19.20109173
58. Fadel R, Morrison AR, Vahia A, Smith ZR, Chaudhry Z, Bhargava P, et al. Early short course corticosteroids in hospitalized patients with COVID-19. *Clin Infect Dis.* (2020) 19:ciaa601. doi: 10.1101/2020.05.04.20074609
59. Keller MJ, Kitsis EA, Arora S, Chen J-T, Agarwal S, Ross MJ, et al. Effect of systemic glucocorticoids on mortality or mechanical ventilation in patients with COVID-19. *J Hosp Med.* (2020) 22:2020. doi: 10.12788/jhm.3497
60. Lu H. Drug treatment options for the 2019-new coronavirus (2019-nCoV). *Biosci Trends.* (2020) 14:69–71. doi: 10.5582/bst.2020.01020
61. World Health Organization. *Novel Coronavirus (2019-nCoV) SITUATION REPORT - 1.* (2020). Available online at: https://www.who.int/docs/default-source/coronaviruse/situation-reports/20200121-sitrep-1-2019-ncov.pdf (accessed July 25, 2020).
62. Li H, Wang YM, Xu JY, Cao B. [Potential antiviral therapeutics for 2019 novel coronavirus]. *Zhonghua Jie He He Hu Xi Za Zhi.* (2020) 43:170–2. doi: 10.3760/cma.j.issn.1001-0939.2020.03.004
63. Cao Y, Deng Q, Dai S. Remdesivir for severe acute respiratory syndrome coronavirus 2 causing COVID-19: An evaluation of the evidence. *Travel Med Infect Dis.* (2020) 35:101647. doi: 10.1016/j.tmaid.2020.101647
64. Elfiky AA. Ribavirin, remdesivir, sofosbuvir, galidesivir, and tenofovir against SARS-CoV-2 RNA dependent RNA polymerase (RdRp): a molecular docking study. *Life Sci.* (2020) 253:117592. doi: 10.1016/j.lfs.2020.117592
65. Jefferson T, Jones MA, Doshi P, Del Mar CB, Hama R, Thompson MJ, et al. Neuraminidase inhibitors for preventing and treating influenza in healthy adults and children. *Cochrane database Syst Rev.* (2014) 2014:CD008965. doi: 10.1002/14651858.CD008965.pub4
66. Yang SNY, Atkinson SC, Wang C, Lee A, Bogoyevitch MA, Borg NA, et al. The broad spectrum antiviral ivermectin targets the host nuclear transport importin α/β1 heterodimer. *Antiviral Res.* (2020) 177:104760. doi: 10.1016/j.antiviral.2020.104760
67. Gupta D, Sahoo AK, Singh A. Ivermectin: potential candidate for the treatment of Covid 19. *Br J Infect Dis.* (2020). doi: 10.1016/j.bjid.2020.06.002
68. Heidary F, Gharebaghi R. Ivermectin: a systematic review from antiviral effects to COVID-19 complementary regimen. *J Antibiot.* (2020) 73:593–602. doi: 10.1038/s41429-020-0336-z
69. Srivatsan P. Potential dual therapeutic approach against SARS-CoV-2/COVID-19 with nitazoxanide and hydroxychloroquine. *Preprint.* (2020).
70. Teoh K-T, Siu Y-L, Chan W-L, Schlüter MA, Liu C-J, Peiris JSM, et al. The SARS coronavirus E protein interacts with PALS1 and alters tight junction formation and epithelial morphogenesis. *Mol Biol Cell.* (2010) 21:3838–52. doi: 10.1091/mbc.e10-04-0338
71. Asgrimsson V, Gudjonsson T, Gudmundsson GH, Baldursson O. Novel effects of azithromycin on tight junction proteins in human airway epithelia. *Antimicrob Agents Chemother.* (2006) 50:1805–12. doi: 10.1128/AAC.50.5.1805-1812.2006
72. Min JY, Jang YJ. Macrolide therapy in respiratory viral infections. *Mediat Inflamm.* (2012) 2012:649570. doi: 10.1155/2012/649570
73. Tran DH, Sugamata R, Hirose T, Suzuki S, Noguchi Y, Sugawara A, et al. Azithromycin, a 15-membered macrolide antibiotic, inhibits influenza A(H1N1)pdm09 virus infection by interfering with virus internalization process. *J Antibiot.* (2019) 72:759–68. doi: 10.1038/s41429-019-0204-x
74. Bosseboeuf E, Aubry M, Nhan T, de Pina JJ, Rolain JM, Raoult D, et al. Azithromycin inhibits the replication of Zika virus. *J Antivir Antiretrovir.* (2018) 10:6–11. doi: 10.4172/1948-5964.1000173
75. Bixler SL, Duplantier AJ, Bavari S. Discovering Drugs for the treatment of ebola virus. *Curr Treat Options Infect Dis.* (2017) 9:299–317. doi: 10.1007/s40506-017-0130-z
76. Gabriels J, Saleh M, Chang D, Epstein LM. Inpatient Use of mobile continuous telemetry for COVID-19 patients treated with hydroxychloroquine and azithromycin. *Hear Case Reports.* (2020) 6:241–3. doi: 10.1016/j.hrcr.2020.03.017
77. Arshad S, Kilgore P, Chaudhry ZS, Jacobsen G, Wang DD, Huitsing K, et al. Treatment with hydroxychloroquine, azithromycin, and combination in patients hospitalized with COVID-19. *Int J Infect Dis.* (2020) 97:396–403. doi: 10.1016/j.ijid.2020.06.099

78. Yao X, Ye F, Zhang M, Cui C, Huang B, Niu P, et al. *In vitro* antiviral activity and projection of optimized dosing design of hydroxychloroquine for the treatment of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). *Clin Infect Dis.* (2020) 71:732–9. doi: 10.1093/cid/ciaa237
79. Hong W. Combating COVID-19 with chloroquine. *J Mol Cell Biol.* (2020) 12:249–50. doi: 10.1093/jmcb/mjaa015
80. Pacheco R, Pachito D, Bagattini A, Riera R. *Hidroxicloroquina e cloroquina para infecção por COVID-19* (2020). Available online at: https://oxfordbrazilebm.com/wp-content/uploads/2020/03/RS_rapida_hidroxi_cloroquina_COVID19.pdf (accessed August 06, 2020).
81. Gao J, Tian Z, Yang X. Breakthrough: Chloroquine phosphate has shown apparent efficacy in treatment of COVID-19 associated pneumonia in clinical studies. *Biosci Trends.* (2020) 14:72–3. doi: 10.5582/bst.2020.01047
82. Monteiro WM, Brito-Sousa JD, Baía-da-Silva D, Melo GC de, Siqueira AM, Val F, et al. Driving forces for COVID-19 clinical trials using chloroquine: the need to choose the right research questions and outcomes. *Rev Soc Bras Med Trop.* (2020) 53:e20200155. doi: 10.1590/0037-8682-0155-2020
83. Tobón C, Palacio LC, Chidipi B, Slough DP, Tran T, Tran N, et al. The antimalarial chloroquine reduces the burden of persistent atrial fibrillation. *Front Pharmacol.* (2019) 10:1392. doi: 10.3389/fphar.2019.01392
84. Rodney R. 2019 Novel Coronavirus (2019-nCoV) Update: Uncoating the Virus. *Am Soc Microbiol.* (2020).
85. *Coronavirus disease 2019 (COVID-19): Epidemiology, Virology, Clinical Features, Diagnosis, and Prevention*. (2020). Available online at: https://www.uptodate.com/contents/coronavirus-disease-2019-covid-19-epidemiology-virology-clinical-features-diagnosis-and-prevention (accessed April 19, 2020).
86. McIntosh K, Hirsh MS, Bloom A. *Coronavirus Disease 2019 (COVID-19): Epidemiology, Virology, Clinical Features, Diagnosis, and Prevention*. (2020). Available online at: https://www.uptodate.com/contents/coronavirus-disease-2019-covid-19-epidemiology-virology-clinical-features-diagnosis-and-prevention (accessed April 19, 2020).
87. Colson P, Rolain JM, Lagier JC, Brouqui P, Raoult D. Chloroquine and hydroxychloroquine as available weapons to fight COVID-19. *Int J Antimicrob Agents.* (2020) 55:105932. doi: 10.1016/j.ijantimicag.2020.105932
88. Chorin E, Dai M, Shulman E, Wadhwani L, Bar-Cohen R, Barbhaiya C, et al. The QT interval in patients with COVID-19 treated with hydroxychloroquine and azithromycin. *Nat Med.* (2020) 26:808–809. doi: 10.1038/s41591-020-0888-2
89. Zhou D, Dai S-M, Tong Q. COVID-19: a recommendation to examine the effect of hydroxychloroquine in preventing infection and progression. *J Antimicrob Chemother.* (2020) 75:1667–70. doi: 10.1093/jac/dkaa114
90. Garraud O, Heshmati F, Pozzetto B, Lefrere F, Girot R, Saillol A, et al. Plasma therapy against infectious pathogens, as of yesterday, today and tomorrow. *Transfus Clin Biol.* (2016) 23:39–44. doi: 10.1016/j.tracli.2015.12.003
91. Sahr F, Ansumana R, Massaquoi TA, Idriss BR, Sesay FR, Lamin JM, et al. Evaluation of convalescent whole blood for treating ebola virus disease in freetown, sierra leone. *J Infect.* (2017) 74:302–9. doi: 10.1016/j.jinf.2016.11.009
92. Mair-Jenkins J, Saavedra-Campos M, Baillie JK, Cleary P, Khaw F-M, Lim WS, et al. The effectiveness of convalescent plasma and hyperimmune immunoglobulin for the treatment of severe acute respiratory infections of viral etiology: a systematic review and exploratory meta-analysis. *J Infect Dis.* (2014) 211:80–90. doi: 10.1093/infdis/jiu396
93. Shen C, Wang Z, Zhao F, Yang Y, Li J, Yuan J, et al. Treatment of 5 critically Ill patients with COVID-19 with convalescent plasma. *JAMA - J Am Med Assoc.* (2020) 323:1582–9. doi: 10.1001/jama.2020.4783
94. Roback JD, Guarner J. Convalescent plasma to treat COVID-19: possibilities and challenges. *JAMA - J Am Med Assoc.* (2020) 16:1561–62. doi: 10.1001/jama.2020.4940
95. Valk SJ, Piechotta V, Chai KL, Doree C, Monsef I, Wood EM, et al. Convalescent plasma or hyperimmune immunoglobulin for people with COVID-19: a rapid review. *Cochrane Database Syst Rev.* (2020) 5:CD013600. doi: 10.1002/14651858.CD013600
96. Li L, Zhang W, Hu Y, Tong X, Zheng S, Yang J, et al. Effect of convalescent plasma therapy on time to clinical improvement in patients with severe and life-threatening COVID-19: A randomized clinical trial. *JAMA*. (2020) 324:1–11. doi: 10.1001/jama.2020.12607
97. Spyropoulos AC, Ageno W, Barnathan ES. Hospital-based use of thromboprophylaxis in patients with COVID-19. *Lancet.* (2020) 395:e75. doi: 10.1016/S0140-6736(20)30926-0
98. Bikdeli B, Madhavan M V., Jimenez D, Chuich T, Dreyfus I, Driggin E, et al. COVID-19 and thrombotic or thromboembolic disease: implications for prevention, antithrombotic therapy, and follow-up: JACC State-of-the-art review. *J Am Coll Cardiol.* (2020) 75:2950–73. doi: 10.1016/j.jacc.2020.04.031
99. Beard JA, Bearden A, Striker R. Vitamin D and the anti-viral state. *J Clin Virol.* (2011) 50:194–200. doi: 10.1016/j.jcv.2010.12.006
100. Kandeel M, Al-Nazawi M. Virtual screening and repurposing of FDA approved drugs against COVID-19 main protease. *Life Sci.* (2020) 251:117627. doi: 10.1016/j.lfs.2020.117627
101. Hemilä H. Vitamin C intake and susceptibility to pneumonia. *Pediatr Infect Dis J.* (1997) 16:836–7. doi: 10.1097/00006454-199709000-00003
102. Nonnecke BJ, McGill JL, Ridpath JF, Sacco RE, Lippolis JD, Reinhardt TA. Acute phase response elicited by experimental bovine diarrhea virus (BVDV) infection is associated with decreased vitamin D and E status of vitamin-replete preruminant calves. *J Dairy Sci.* (2014) 97:5566–79. doi: 10.3168/jds.2014-8293
103. Lippi G, Henry BM, Bovo C, Sanchis-Gomar F. Health risks and potential remedies during prolonged lockdowns for coronavirus disease 2019 (COVID-19). *Diagnosis.* (2020) 7:85–90. doi: 10.1515/dx-2020-0041
104. Teymoori-Rad M, Shokri F, Salimi V, Marashi SM. The interplay between vitamin D and viral infections. *Rev Med Virol.* (2019) 29:e2032. doi: 10.1002/rmv.2032
105. Jiménez-Sousa M ángeles, Martínez I, Medrano LM, Fernández-Rodríguez A, Resino S. Vitamin D in human immunodeficiency virus infection: influence on immunity and disease. *Front Immunol.* (2018) 9:458. doi: 10.3389/fimmu.2018.00458
106. Havers F, Smeaton L, Gupte N, Detrick B, Bollinger RC, Hakim J, et al. 25-Hydroxyvitamin D insufficiency and deficiency is associated with HIV disease progression and virological failure post-antiretroviral therapy initiation in diverse multinational settings. *J Infect Dis.* (2014) 210:244–53. doi: 10.1093/infdis/jiu259
107. Gruber-Bzura BM. Vitamin D and influenza—Prevention or therapy? *Int J Mol Sci.* (2018) 19:2419. doi: 10.3390/ijms19082419
108. Searing DA, Zhang Y, Murphy JR, Hauk PJ, Goleva E, Leung DYM. Decreased serum vitamin D levels in children with asthma are associated with increased corticosteroid use. *J Allergy Clin Immunol.* (2010) 125:995–1000. doi: 10.1016/j.jaci.2010.03.008
109. Martineau AR, Jolliffe DA, Hooper RL, Greenberg L, Aloia JF, Bergman P, et al. Vitamin D supplementation to prevent acute respiratory tract infections: systematic review and meta-analysis of individual participant data. *BMJ.* (2017) 356:i6583. doi: 10.1136/bmj.i6583
110. Muscogiuri G, Barrea L, Savastano S, Colao A. Nutritional recommendations for CoVID-19 quarantine. *Eur J Clin Nutr.* (2020) 74:850–1. doi: 10.1038/s41430-020-0635-2
111. Watkins J. Preventing a covid-19 pandemic. *BMJ.* (2020) 368:m810. doi: 10.1136/bmj.m810
112. Fabbri A, Infante M, Ricordi C. Editorial – vitamin D status: a key modulator of innate immunity and natural defense from acute viral respiratory infections. *Eur Rev Med Pharmacol Sci.* (2020) 24:4048–52. doi: 10.26355/eurrev_202004_20876
113. Maares M, Haase H. Zinc and immunity: an essential interrelation. *Arch Biochem Biophys.* (2016) 611:58–65. doi: 10.1016/j.abb.2016.03.022
114. Valeska S, De B, Silva F, Morgane M, Souza1 F-RC, Raque De Santana J, et al. Child zinc brain. *Am J Clin Nutr.* (2020) 2:123–35. doi: 10.1093/ajcn/68.2.464S
115. Read SA, Obeid S, Ahlenstiel C, Ahlenstiel G. The Role of zinc in antiviral immunity. *Adv Nutr.* (2019) 10:696–710. doi: 10.1093/advances/nmz013
116. te Velthuis AJW, van den Worm SHE, Sims AC, Baric RS, Snijder EJ, van Hemert MJ. Zn2+ inhibits coronavirus and arterivirus RNA

Polymerase activity *in vitro* and zinc ionophores block the replication of these viruses in cell culture. *PLoS Pathog.* (2010) 6:e1001176. doi: 10.1371/journal.ppat.1001176

117. Singh M, Das RR. Zinc for the common cold. *Cochrane Database Syst Rev.* (2013) 2013:CD001364. doi: 10.1002/14651858.CD001364.pub4
118. Zhang L, Liu Y. Potential interventions for novel coronavirus in China: a systematic review. *J Med Virol.* (2020) 92:479–90. doi: 10.1002/jmv.25707
119. Xue J, Moyer A, Peng B, Wu J, Hannafon BN, Ding WQ. Chloroquine is a zinc ionophore. *PLoS ONE.* (2014) 9:e109180. doi: 10.1371/journal.pone.0109180

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cell and Developmental Biology

REVIEW
published: 16 September 2020
doi: 10.3389/fcell.2020.559841

Insights on SARS-CoV-2 Molecular Interactions With the Renin-Angiotensin System

Larissa Braga Costa†, Lucas Giandoni Perez†, Vitória Andrade Palmeira, Thiago Macedo e Cordeiro, Victor Teatini Ribeiro, Katharina Lanza‡ and Ana Cristina Simões e Silva‡*

Department of Pediatrics, Interdisciplinary Laboratory of Medical Investigation, Faculty of Medicine, Federal University of Minas Gerais (UFMG), Belo Horizonte, Brazil

OPEN ACCESS

Edited by:
Tatsuo Shioda, Osaka University, Japan

Reviewed by:
Robert C. Speth, Nova Southeastern University, United States
Dulce Elena Casarini, Federal University of São Paulo, Brazil

***Correspondence:**
Ana Cristina Simões e Silva
acssilva@hotmail.com

†*These authors have contributed equally to this work*

‡*These authors have contributed equally to this work and share senior authorship*

Specialty section:
This article was submitted to Molecular Medicine, a section of the journal Frontiers in Cell and Developmental Biology

Received: *07 May 2020*
Accepted: *25 August 2020*
Published: *16 September 2020*

Citation:
Costa LB, Perez LG, Palmeira VA, Macedo e Cordeiro T, Ribeiro VT, Lanza K and Simões e Silva AC (2020) Insights on SARS-CoV-2 Molecular Interactions With the Renin-Angiotensin System. Front. Cell Dev. Biol. 8:559841. doi: 10.3389/fcell.2020.559841

The emergence of SARS-CoV-2/human/Wuhan/X1/2019, a virus belonging to the species *Severe acute respiratory syndrome-related coronavirus,* and the recognition of Coronavirus Disease 2019 (COVID-19) as a pandemic have highly increased the scientific research regarding the pathogenesis of COVID-19. The Renin Angiotensin System (RAS) seems to be involved in COVID-19 natural course, since studies suggest the membrane-bound Angiotensin-converting enzyme 2 (ACE2) works as SARS-CoV-2 cellular receptor. Besides the efforts of the scientific community to understand the virus' molecular interactions with human cells, few studies summarize what has been so far discovered about SARS-CoV-2 signaling mechanisms and its interactions with RAS molecules. This review aims to discuss possible SARS-CoV-2 intracellular signaling pathways, cell entry mechanism and the possible consequences of the interaction with RAS components, including Angiotensin II (Ang II), Angiotensin-(1-7) [Ang-(1-7)], Angiotensin-converting enzyme (ACE), ACE2, Angiotensin II receptor type-1 (AT1), and Mas Receptor. We also discuss ongoing clinical trials and treatment based on RAS cascade intervention. Data were obtained independently by the two authors who carried out a search in the PubMed, Embase, LILACS, Cochrane, Scopus, SciELO and the National Institute of Health databases using Medical Subject Heading terms as "SARS-CoV-2," "COVID-19," "Renin Angiotensin System," "ACE2," "Angiotensin II," "Angiotensin-(1-7)," and "AT1 receptor." Similarly to other members of *Coronaviridae* family, the molecular interactions between the pathogen and the membrane-bound ACE2 are based on the cleavage of the spike glycoprotein (S) in two subunits. Following the binding of the S1 receptor-binding domain (RBD) to ACE2, transmembrane protease/serine subfamily 2 (TMPRSS2) cleaves the S2 domain to facilitate membrane fusion. It is very likely that SARS-CoV-2 cell entry results in downregulation of membrane-bound ACE2, an enzyme that converts Ang II into Ang-(1-7). This mechanism can result in lung injury and vasoconstriction. In addition, Ang II activates pro-inflammatory cascades when binding to the AT1 Receptor. On the other

hand, Ang-(1-7) promotes anti-inflammatory effects through its interactions with the Mas Receptor. These molecules might be possible therapeutic targets for treating COVID-19. Thus, the understanding of SARS-CoV-2 intracellular pathways and interactions with the RAS may clarify COVID-19 physiopathology and open perspectives for new treatments and strategies.

Keywords: Renin Angiotensin System, SARS-CoV-2, ACE2, COVID-19, Ang II, Ang-(1-7), AT1 receptor, pathogenesis

INTRODUCTION

SARS-CoV-2/human/Wuhan/X1/2019 was firstly described in December 2019, in Wuhan, China (Zhou et al., 2020). The World Health Organization (WHO) declared the Coronavirus Disease 2019 (COVID-19) pandemic on 11 March 2020. The scientific community is concentrating efforts to better understand COVID-19 natural course, as well as its pathogenesis and possible therapeutic strategies. SARS-CoV-2 is the etiological agent of COVID-19 and belongs to the *Severe Acute Respiratory Syndrome-related coronavirus* species (Coronaviridae Study Group of the International Committee on Taxonomy of Viruses, 2020), which are RNA-enveloped viruses from the *Coronaviridae* family.

Besides SARS-CoV-2, only SARS-CoV and MERS-CoV outbreaks, in 2003 and in 2012 have been described to cause the life threatening diseases, the Severe Acute Respiratory Syndrome (SARS) and the Middle East Respiratory Syndrome (MERS), respectively. From 1965, when the first coronavirus was identified in patients with common cold (Tyrrell and Bynoe, 1965) until now, seven coronaviruses are described to cause human diseases: HCoV-OC43, HKU1, HCoV-229E, HCoV-NL63, SARS-CoV, MERS-CoV, and SARS-CoV-2 (Wang Q. et al., 2020). These pathogens are zoonotic viruses that jumped species boundaries (Zhang and Holmes, 2020), leading to human diseases. The coronaviruses share the potential to outbreak as pandemics, but small and crucial genetic mutations directly influence their infectivity. On this wise, viruses are obligate intracellular pathogens and their survival relies entirely on host cell machinery control to synthesize and organize their structural components. Viral infections are complex processes, which initiate with viral recognition and attachment to the host cell receptor. SARS-CoV and SARS-CoV-2 have several genetic similarities (Lu et al., 2020; Zhang and Holmes, 2020) and both attach to Angiotensin-converting Enzyme 2 (ACE2), which is anchored to plasma membrane via its transmembrane domain (Hoffmann et al., 2020; Wan et al., 2020). SARS-CoV-2 envelope is composed of two proteins to structure maintenance (membrane and envelope proteins), and the spike glycoprotein (S), which mediates host cell entry. SARS-CoV-2 entry mechanisms are still under investigation and further characterization is needed to best describe SARS-CoV-2 hypothetical mechanisms. In this review, we present published studies about SARS-CoV-2, SARS-CoV, ACE2 and other mediator components of the first step of infection. Furthermore, we also show how the binding of SARS-CoV-2 may trigger a Renin Angiotensin System (RAS) imbalance due to its binding to ACE2, possibly contributing to the pathogenesis of COVID-19.

Angiotensin-converting Enzyme 2 is an important component of the RAS (Donoghue et al., 2000; Tipnis et al., 2000) a molecular system composed of a wide range of peptides, enzymes, and receptors (Simões e Silva and Flynn, 2012). Angiotensin II (Ang II) and Angiotensin-(1-7) [Ang-(1-7)] are the major effector molecules of the two main RAS pathways: the classical axis, composed by the Angiotensin-converting enzyme (ACE), Ang II and Angiotensin II receptor type 1 (AT1), and the alternative axis, which includes ACE2, Ang-(1-7), and the Mas receptor (MasR) (Santos et al., 2003). Under physiological circumstances, the homeostatic state is achieved due to the counter-regulatory actions of these two arms. Although there's hardly any published evidence in this regard, SARS-CoV-2 binding to ACE2 might result in ACE2 availability reduction, leading to ACE2/Ang-(1-7)/MasR axis downregulation and consequent exacerbation of the ACE/Ang II/AT1R axis (Rodrigues Prestes et al., 2017; Lanza et al., 2020). This may cause important pulmonary, immune and hematological disturbances. Thus, RAS imbalance might not only be a consequence of the disease, but a crucial step of COVID-19 pathogenesis (Lanza et al., 2020).

This counter-regulatory pattern might explain both symptomatology and epidemiological patterns of risk groups. Usually after a 5-day incubation period, the most common observed symptoms are fever, dry cough, tiredness, and neurological manifestations, including anosmia, ageusia and dysgeusia (Lechien et al., 2020; Li Q. et al., 2020). Other signs and symptoms can also be found, including sputum production, headache, hemoptysis, diarrhea, dyspnea, lymphopenia and important changes in lung imaging investigation (Rothan and Byrareddy, 2020). Evidence shows that the symptoms related to severe pneumonia are mainly due to an exaggerated immune response and cytokine storm (Mehta et al., 2020). These findings are closely related to pulmonary tissue damage, inflammatory response and hematological disturbances. The three steps pathophysiology proposed in this article link these phenomena with the RAS imbalance hypothesis (Lanza et al., 2020).

METHODS

The references were obtained independently by the two authors, who carried out a comprehensive and non-systematic search in the PubMed, Embase, LILACS, Cochrane, Scopus and SciELO databases. Search strategies included Medical Subject Heading

terms as: "SARS-CoV-2," "COVID-19," "Renin Angiotensin System," "ACE2," "Angiotensin II," "Angiotensin-(1-7)," and "AT1 receptor." The search emphasized recent articles, published case series, consensus statements, guidelines, meta-analyses, systematic reviews and prospective cohort studies, critically reviewed and selected by the authors.

SIGNALING AND CELL ENTRY MECHANISMS OF SARS-CoV-2

Summarizing Current Knowledge About How SARS-CoV-2 Enters Host Cell

Several studies reported membrane-bound ACE2 as SARS-CoV-2 receptor (Hoffmann et al., 2020; Liu et al., 2020; Zhou et al., 2020). The binding of SARS-CoV-2 to its functional receptor, the membrane-bound ACE2, facilitates the virus entry into the cell. Viral binding to ACE2 involves distinct domains of the spike (S) protein. Due to a multi-step variation of its conformational state, SARS-CoV-2 is able to attach itself to the cell surface, firmly binding to ACE2 and starting the membrane fusion step (Letko et al., 2020). Other membrane proteins are essential to viral entry into the cell through priming and activating of the S protein. Firstly, SARS-CoV-2 has a FURIN cleavage site, which is absent in SARS-CoV (Coutard et al., 2020). This site may enhance binding affinity between the SARS-CoV-2 S protein and the human membrane-bound ACE2. Moreover, SARS-CoV-2 receptor-binding domain (RBD) has significant differences in its amino acid sequence if compared to SARS-CoV RBD, leading to higher affinity of SARS-CoV-2 to membrane-bound ACE2. The binding of SARS-CoV spike protein occurs with less affinity, due to its naturally less accessible conformation (Shang et al., 2020a). Additionally, SARS-CoV-2 exploits a cellular serine protease, TMPRSS2, and, in a smaller rate, an endosomal cysteine protease, cathepsin B, and L (CatB/L) (Hoffmann et al., 2020). In this sense, the TMPRSS2 downregulation as a cell self-defense mechanism (Guzzi et al., 2020) may be overpassed by SARS-CoV-2 through the CatB/L endosomal pathway (Kawase et al., 2012). Simultaneous treatment *in vitro* with a serine protease inhibitor and a cathepsin inhibitor blocks both cell entry and the multistep growth of SARS-CoV-2 in human airway epithelial cells (Hoffmann et al., 2020).

Although the endocytosis-mediated entry is not a consensus, growing evidence points to mechanisms for this pathway. Endosomal transport through the cell depends on H^+ - ATPases activity, which are coupled to the endosomal membrane due to the fusion of circulating vesicles carrying these proton pumps (Bright et al., 2016; Naslavsky and Caplan, 2018). Subsequently, endosomes can fuse with lysosomes. In the meantime, internal pH decreases, inducing irreversible conformational changes by a variety of mechanisms, including protonation of histidine residues and salt bridges. Furthermore, endosomal cathepsin L proteolysis might act as a third priming event (Simmons et al., 2005). This buries S fusion peptide (FP) and exposes it to the endosomal membrane (Rachakonda et al., 2007; White et al., 2008). The endosomal membrane is then disrupted, forming a pore through which the viral particles translocate to the cytoplasm.

SARS-CoV-2 and S Protein Structure

The SARS-CoV-2 is an enveloped, positive-strand RNA *Betacoronavirus* of the Coronaviridae family. Genomic sequence analysis of SARS-CoV-2 suggests that the 30 kb genome encodes as many as 14 open reading frames (ORFs) (Gordon et al., 2020). ORF1a/ORF1ab encodes 16 non-structural proteins (Nsp1-16) that form the replicase and transcriptase complex (RTC). The other 13 encode four structural proteins – Spike (S), Envelope (E), Membrane (M), and Nucleocapsid (N) – and nine putative accessory factors.

There are six open reading frame proteins (ORFs), ORF3a, ORF6, ORF7a, ORF7b, ORF8, and ORF10, and the polyprotein ORF1ab (Srinivasan et al., 2020). A study of evolutionary conservation found that the majority of these proteins has either no modifications or a mutation in the peripheral binding region, in comparison to SARS-CoV (Srinivasan et al., 2020). However, unlike most of these protein cited before, the Spike protein of SARS-CoV-2 has significant changes at amino acid sequence if compared to other human coronaviruses (hCoV), including SARS-CoV (Ou et al., 2020; Shang et al., 2020b; Walls et al., 2020; Wang Q. et al., 2020). S is formed by a S1 subunit, responsible for receptor binding, attached to S2, the subunit responsible for membrane fusion, which comprises three subdomains that loop back on each other (Shang et al., 2020a). This might result in essential differences regarding molecular interactions, which enhance the binding affinity of SARS-CoV-2 to ACE2.

Although all viral fusion proteins have a similar conformation at the end of virus-cell membrane fusion, known as trimer of hairpins, they are divided into three classes that differ in structure (White et al., 2008). Coronaviruses S proteins are class I viral fusion proteins, which mean that these proteins are assembled into trimers in their pre- and post-fusion states (White et al., 2008). The trimer of hairpins form a structure called 6HB, a six-helix bundle that approximates target cell and virion membranes (White et al., 2008). Viral S proteins have two functional subunits: S1, the distal receptor-binding subunit, and S2, the fusion machinery subunit (Millet and Whittaker, 2015). In addition, S1 has the RBD and N-linked glycans, which may function as glycan shields, protecting SARS-CoV-2 from antibody recognition (Walls et al., 2020). S1 is the less conserved subunit, reflecting a high selective pressure promoted by the host immune system. SARS-CoV and SARS-CoV-2 S share about 79.6% of amino acid residue sequence in regard to the entire protein, but only 74% comparing the RBD (Ou et al., 2020). On the other hand, S2 has a high sequence identity with other hCoV in some important regions: the short cytosolic tail; palmitoylated cysteines-transmembrane domain; two heptad repeats (HR1 and HR2); and a fusion peptide, a hydrophobic amino acid residue sequence that engages target membrane (White et al., 2008; Millet and Whittaker, 2015; Walls et al., 2020; **Figure 1**). S1/S2 and S2′ are the two cleavage sites in SARS-CoV-2 S, allowing the activation and priming steps (Hoffmann et al., 2020) through

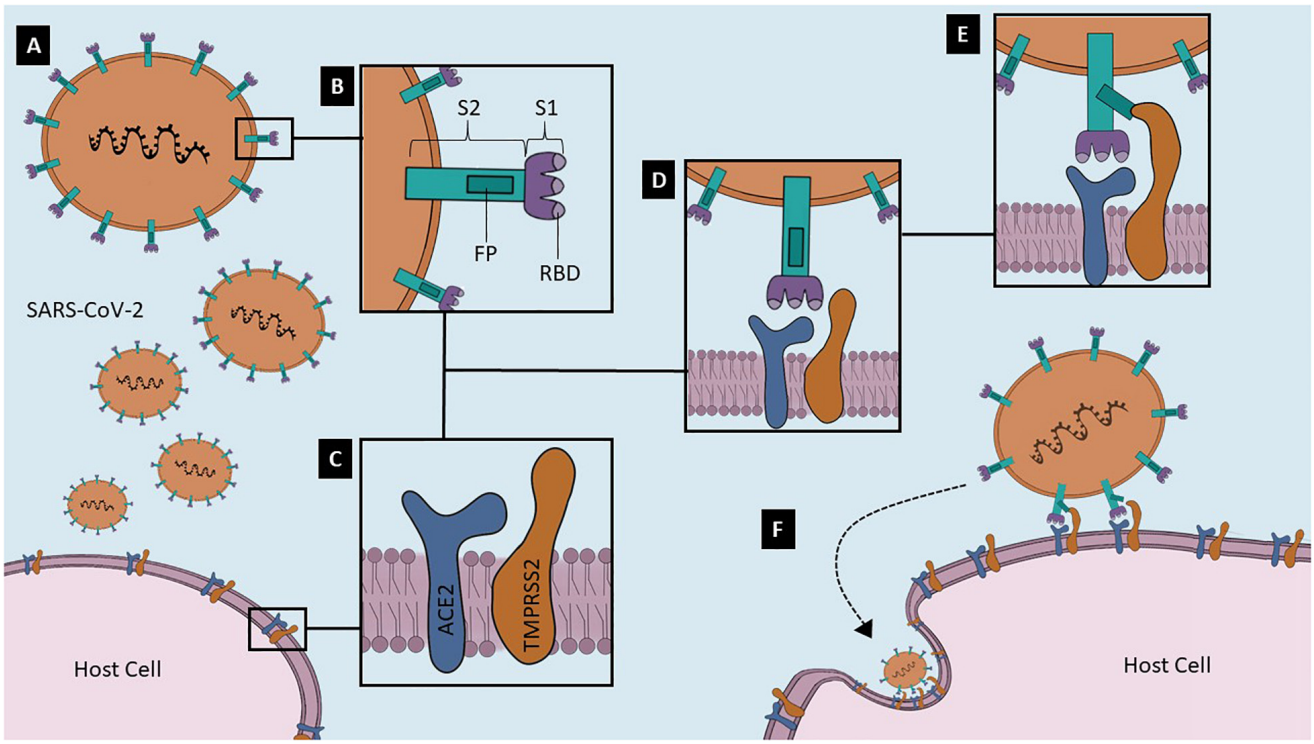


FIGURE 1 | Potential mechanisms of SARS-CoV-2 Spike glycoprotein (S) binding to and invading host cell. **(A)** Virion gets closer to the host cell that expresses a high-affinity binding receptor on its surface. In its native fusion-competent state, the Spike glycoprotein (S) of SARS-CoV-2 is anchored on the virion envelope. **(B)** S is formed by a S1 subunit, which contains the receptor-binding domain (RBD), and by a S2 subunit, comprising the fusion peptide (FP) domain. **(C)** Two of the most important proteins in the host cell surface related to the virus entry are Angiotensin-converting Enzyme 2 (ACE2) and Transmembrane protease/serine subfamily 2 (TMPRSS2); **(D)** Membrane bound-ACE2 is the SARS-CoV-2 receptor, and TMPRSS2 is the S primer. These two host cell proteins probably form a complex on the lipid bilayer; **(E)** S1 RBD attaches to ACE2 and TMPRSS2 cleaves S2 in a step named as priming, leading to the exposure of the FP; **(F)** Viral S protein is anchored not only to virion surface, but also to host plasma membrane. Hence, other S is recruited and the endocytic viral entry process begins. These are representative no-scale images.

protease actions. S1/S2 also has a four amino acid residue furin-like cleavage sequence (PRRA), indicating that furin, a calcium-dependent serine peptidase, can cleave this site (White et al., 2008; Walls et al., 2020). S2′ contains a residue sequence located upstream the fusion peptide and can be cleaved by a diversity of proteases (Hoffmann et al., 2020; Walls et al., 2020). Although few evidence show the role of these proteolytic activation sites on SARS-CoV-2 life cycle, it is believed that S2′ is cleaved during biosynthesis and S1/S2 is cleaved for the virus to enter the cell (Walls et al., 2020).

S Protein Binding to ACE2

Angiotensin-converting enzyme 2 is a zinc membrane-bound metalloproteinase that acts as a carboxypeptidase able to hydrolyze Ang I to Ang-(1-9) and Ang II to Ang-(1-7). ACE converts Ang I into Ang II, while ACE2 forms Ang-(1-9) from Ang I. ACE2 differs from ACE regarding its insensitivity to conventional ACE inhibitors (Donoghue et al., 2000; Tipnis et al., 2000; Rodrigues Prestes et al., 2017). Both SARS-CoV and SARS-CoV-2 have ACE2 as their host cell receptor (Lee et al., 2003; Letko et al., 2020), but SARS-CoV-2 S RBD has a receptor binding motif (RBM) capable to attach ACE2 with higher affinity if compared to SARS-CoV S. Studies showed major structural changes that explain this difference (Glowacka et al., 2010; Shang et al., 2020b; Walls et al., 2020; Wrapp et al., 2020): (1) variable ridge loop with four residue motif (Gly-Gln-Thr-Gly) instead of a three motif, allowing additional main-hydrogen bonding between RBM and ACE2 N-terminal helix; (2) SARS-CoV-2 S RBM better insertion into ACE2 hydrophobic pocket, due to novel interactions because of Leu472; (3) Lys31 and Glu35 from ACE2 binding to Leu455 and Gln493 from SARS-CoV-2, respectively (hotspot 31); (4) unique hydrogen bonding between Lys353 from ACE2 and SARS-CoV-2 RBD main chain (hotspot 353) (Shang et al., 2020b). Hence, the high-affinity SARS-CoV-2/ACE2 interaction could be an explanation for the greater infectivity of this virus when compared to others hCoVs.

Figure 1 illustrates the mechanisms of SARS-CoV-2 binding to membrane-bound ACE2 and subsequent entry into the human cell.

Other Potential SARS-CoV-2 Receptors

Tissue tropism and host range are determined by a variety of factors. Nonetheless, receptor recognition and attachment are essential steps for a viral infection (Maginnis, 2018).

Intracellular pathogens usually attach to more than one host cell surface structure that exerts the function of viral receptor. Carbohydrates, such as sialic acid (SiAc), and proteins, integrins and the membrane-bound ACE2 for instance, are common receptors used by viruses. Studies suggest that infection might follow a series of receptor engaging and detachment, until the pathogen interacts with host cells in a high-affinity event (Maginnis, 2018).

Sialic acid is ubiquitously expressed on the surface of host cells and is capable of mediating cell adhesion and transduction-signaling events. SiAc might be the first host cell-virus interaction in SARS-CoV-2 infection. MERS-CoV binds not only to dipeptidyl peptidase 4 (DPP4), its protein receptor, but also to sialic acids (α2,3-linked especially) (Li et al., 2017). Furthermore, SARS-CoV-2 is the first discovered hCoV that has a specific motif in its S able to bind to integrins receptors, integral membrane proteins arranged as heterodimers with alpha and beta subunits. Integrins mediate a variety of mechanisms, including cell adhesion, signaling events, and cytoskeletal rearrangement. In relation to the SARS-CoV-2, these molecules recognize two specific motifs: RGD (Arg-Gly-Asp) and KGE (Lys-Gly-Glu). Although integrin-binding is essential for a variety of human-viruses (Stewart and Nemerow, 2007), no previously described coronaviruses were capable of making these transmembrane proteins as receptors. Therefore, SARS-CoV-2 is the first coronavirus that has a RGD motif S protein present in the RBD of S1 (residues 403–405) (Sigrist et al., 2020). The implications of this finding, however, are still unknown.

The extracellular matrix metalloproteinase inducer (EMMPRIN), also known as CD147, represents another potential receptor for SARS-CoV-2 (Ulrich and Pillat, 2020). Wang K. et al. (2020) showed that SARS-CoV-2 invaded human host cells via CD147 binding (Wang K. et al., 2020). This protein belongs to an immunoglobulin superfamily enrolled in inflammatory processes and virus host cell entry (Pushkarsky et al., 2001; Chen et al., 2005; Sagkan and Akin-Bali, 2020). Differently from ACE2, CD147 is ubiquitously expressed in epithelium and immune cells (Radzikowska et al., 2020). Interestingly, CD147 is upregulated in patients with obesity and diabetes, which might explain, at least in part, why these comorbidities are considered risk factors for severe COVID-19.

Proteases and Priming

Proteolytic activation of viral fusion-protein is an essential step for membrane fusion in a variety of viruses. Besides allowing the fusion triggering process named as priming, induced viral fusion-protein conformational changes release sufficient energy to overcome the lipid bilayer fusion energy barrier. On that matter, SARS-CoV-2 receptor is a protease distinct of the S primer, once RBD binds ACE2 distant from its action site (Shang et al., 2020b). Therefore, several proteases are capable of priming viral fusion-proteins. Based on previous knowledge about other hCoVs, researchers discovered that TMPRSS2 is essential for SARS-CoV-2 cell entry (Hoffmann et al., 2020). TMPRSS2 belongs to the type II transmembrane serine proteases family (TTSP) and is found on cell surface or in the secretory pathway (Szabo and Bugge, 2008, 2011). Evidence suggests that this protease forms complexes with ACE2 in plasma membrane microdomains (Shulla et al., 2011; Tarnow et al., 2014), corroborating to the hypothesis that TMPRSS2 and ACE2 operate together in SARS-CoV-2 cell entry. Thus, TMPRSS2 might catalyze the cleavage of S2′ after S binding to the receptor.

Cathepsin L, another protease related to a variety of coronaviruses, seems also to be involved in SARS-CoV-2 molecular mechanisms (Ou et al., 2020). This ubiquitously expressed protein is a cysteine protease activated in low pH. Besides acting as an essential protease for several viruses (Millet and Whittaker, 2015), cathepsin L can be found in lysosomes (Turk et al., 2012). SARS-CoV and MERS-CoV, for instance, used cathepsin L to cell entry (Simmons et al., 2005; Qian et al., 2013). The role of cathepsin L in SARS-CoV-2 cell infection is not well defined. Bosch et al. (2008) reported that cathepsin L cleavage site for SARS-CoV S is T678, 11 residues downstream the trypsin cleavage site.

RENIN-ANGIOTENSIN SYSTEM AND COVID-19

RAS Ubiquitous Role in Homeostasis

The Renin-Angiotensin System was first conceived as centered in the local and systemic actions of Angiotensin II (Ang II). However, last decades' studies regarding the RAS established a complex and dynamic molecular cascade with two fundamental arms, a classical and an alternative axes, and a range of counter-regulatory actions in different organ systems. Its endocrine (tissue-to-tissue), paracrine (cell-to-cell) and intracrine (intracellular/nuclear) effects (Patel et al., 2017) are crucial for cardiovascular, renal, immune, pulmonary, and neural homeostasis (Nehme et al., 2015). The RAS also plays a pivotal role in several pathophysiological disease models, including pulmonary and renal diseases (Simões e Silva and Flynn, 2012; Magalhaes et al., 2018).

The classical axis, comprising the ACE, its main product Ang II and the angiotensin II type 1 (AT1) receptor, mediates the well described body fluid homeostasis through restoration of blood volume. Several lines of evidence, however, attribute a range of deleterious actions to the classical ACE/Ang II/AT1R axis, including enhancement of inflammation, fibrosis, cellular growth, and migration (Rodrigues Prestes et al., 2017). Additionally, this axis triggers vasoconstriction, cardiac hypertrophy and reactive oxygen species (ROS) production (Ardaillou, 1998; Yamada et al., 1998).

Findings on the ACE2/Ang-(1-7)/MasR arm are substantially new. Santos et al. (1988) described Ang-(1-7) production in dog brainstem dependently and independently of Ang II formation, which suggested an alternative route for generating RAS fragments. In the same year, Schiavone et al. (1988) showed the first biological effect of Ang-(1-7), with the heptapeptide was able to release Vasopressin from pituitary-hypothalamus tissue explants (Schiavone et al., 1988). Further, several actions of Ang-(1-7) were successively reported (Santos et al., 2000). However, until 2000, the preferential route of Ang-(1-7) formation was still lacking. In this regard, in 2000, two independent research groups

discovered almost simultaneously the enzyme homolog to ACE, named ACE2, as the main responsible for the conversion of Ang II into Ang-(1-7) (Donoghue et al., 2000; Tipnis et al., 2000). Three years later, Santos et al. (2003) determined the G-protein coupled Mas receptor (MasR) as Ang-(1-7) receptor. The high affinity binding of Ang-(1-7) to the MasR is possible after the cleavage of Ang II by ACE2, subtracting the Phenylalanine amino acid (Santos et al., 2018). Discoveries on ACE2 and the MasR resulted in a new conception of the RAS. Currently, the RAS is defined as a dual arm system formed by two counter-regulatory axes, the classical ACE/Ang II/AT1 axis and the alternative ACE2/Ang-(1-7)/MasR arm (Santos et al., 2005). The alternative arm exerts beneficial effects through counter-regulating the RAS classical axis and its effects includes vasodilation, inhibition of cell growth and ROS production, anti-inflammatory, anti-thrombosis and anti-arrhythmogenic actions (Simões e Silva and Flynn, 2012; Simões e Silva and Teixeira, 2016; Rodrigues Prestes et al., 2017).

Several studies demonstrated the pivotal role of RAS imbalance in disease progression through disruption of the system's equilibrium (Simões e Silva and Teixeira, 2016). Hence, the reduction of a RAS molecule function or bioavailability might lead to exacerbation of one axis. Indeed, administration of RAS blocker therapy, which inhibits the classical RAS axis, has been also described to enhance the alternative axis in humans and animal models (Simões e Silva and Flynn, 2012). This principle might be an underlying component in many diseases pathogenesis, including COVID-19 (Lanza et al., 2020). **Figure 2** shows the potential mechanisms that may link RAS imbalance to signs and symptoms of COVID-19.

RAS Imbalance and COVID-19 Pathophysiology

Growing evidence supports the role of ACE2 downregulation in COVID-19 pathophysiology and the possible contribution of RAS axes unbalance to COVID-19 natural history (D'Ardes et al., 2020; Gheblawi et al., 2020; Lanza et al., 2020; Verdecchia et al., 2020). It seems that the dynamics of ACE2 and SARS-CoV-2 infection relies on an apparent paradox that depends on the bioavailability of ACE2: either (1) the individual infected by the virus has enough reservoir of ACE2 to resist the depletion of the enzyme and still counteract the deleterious effects of reduced ACE2 levels and activity; or (2) individuals with a small reservoir of ACE2 will not be able to activate the anti-inflammatory axis of the RAS and consequently will suffer from an exacerbated activation of the classical pro-inflammatory arm.

Angiotensin-converting enzyme 2 is significantly downregulated in several experimental studies with induced pulmonary injury (Imai et al., 2005; Kuba et al., 2006; Gu et al., 2016; Sodhi et al., 2019). Its blockade or genetic manipulation resulted in enhanced vascular permeability, neutrophil accumulation, increased lung edema, and worsened lung function in a study conducted by Kuba et al. (2006). Gu et al. (2016) observed reduced animal survival and exacerbated lung injury following respiratory syncytial viral infection in mice with genetic deletion of ACE2 gene. Another study in a mice model of *Pseudomonas aeruginosa* lung infection showed an increased pro-inflammatory cytokine and chemokine response, as well as parenchymal inflammation (Sodhi et al., 2019). In the pathogenesis of SARS-CoV infection specifically, Imai et al. (2005) attributed to Ang II upregulation the responsibility for severe lung failure via AT1.

The diminished ACE2 levels may result in: (1) Ang II upregulation, leading to classical RAS axis overactivity; and (2) Ang-(1-7) depletion, attenuating the protective effects of the alternative RAS axis (**Figure 2**). Ang II is a pro-inflammatory peptide and its upregulation contributes to acute lung injury by promoting endothelial dysfunction and cytokine storm (Rodrigues Prestes et al., 2017). The unbalance between both RAS axes may result in three major disturbances: pulmonary, inflammatory/immune, and hematological. Therefore, as shown in **Figure 2**, a three-phase disease course was proposed in order to explain COVID-19 pathophysiology and correlate its evolution to RAS activity (Lanza et al., 2020).

First phase: ACE2 is expressed in several tissues, including airways epithelium, brain, bone marrow, gastrointestinal (GI) tract, kidney, and heart (Nehme et al., 2015; Li M. Y. et al., 2020). Airways epithelium is the initial site of SARS-CoV-2 infection, from where the virus spreads throughout the body. Therefore, early in the disease course, COVID-19 is remarkably distinguished from other respiratory diseases due to the lymphopenia found in nearly half of the patients at admission (Rodriguez-Morales et al., 2020; Tan et al., 2020). Several possible explanations support this finding, with the four most promising ideas being related to the invasion of different tissues and immune activation. The first one is the invasion of bone marrow. ACE2 is found in hematopoietic stem/progenitor cells (HSPC) and viral induced hypoxia might lead to three main consequences: (1) increased proliferation and migration of HSPC; (2) upregulation of ACE2 and Mas receptor; and (3) shedding of ACE2 ectodomain in HSPC (Joshi et al., 2019). The second hypothesis regards lymphocyte invasion, as this cell expresses ACE2 on its surface and the novel coronavirus is able to invade this cell. The third one considers the deflagration of the hemophagocytic lymphohistiocytosis (HLH), although this mechanism is only enhanced later in disease's course and might be related to other clinical manifestations. Lastly, studies hypothesized on SARS-CoV-2 interactions with the GI tract, as the function of ACE2 in this system is still unclear and GI symptoms are common in COVID-19 (Li M. Y. et al., 2020; Musa, 2020; Tan et al., 2020).

Second phase: the second stage in COVID-19 is the pulmonary involvement. In this regard, Ang-(1-7) depletion might prosecute a key role, given that Ang-(1-7) allegedly reduces lung inflammation, fibrosis, and pulmonary arterial hypertension (Jia, 2016; Santos et al., 2018). In COVID-19 cases with severe manifestations, the disease does not behave as a typical acute respiratory distress syndrome (ARDS) (Gattinoni et al., 2020b). Therefore, the described phenomenon of severe hypoxemia in compliant lungs may be due to a low ventilation-perfusion ratio as a result of lost perfusion regulation and hypoxic vasoconstriction reaction (Gattinoni et al., 2020a; Wang D. et al., 2020). This underlying pathophysiology may

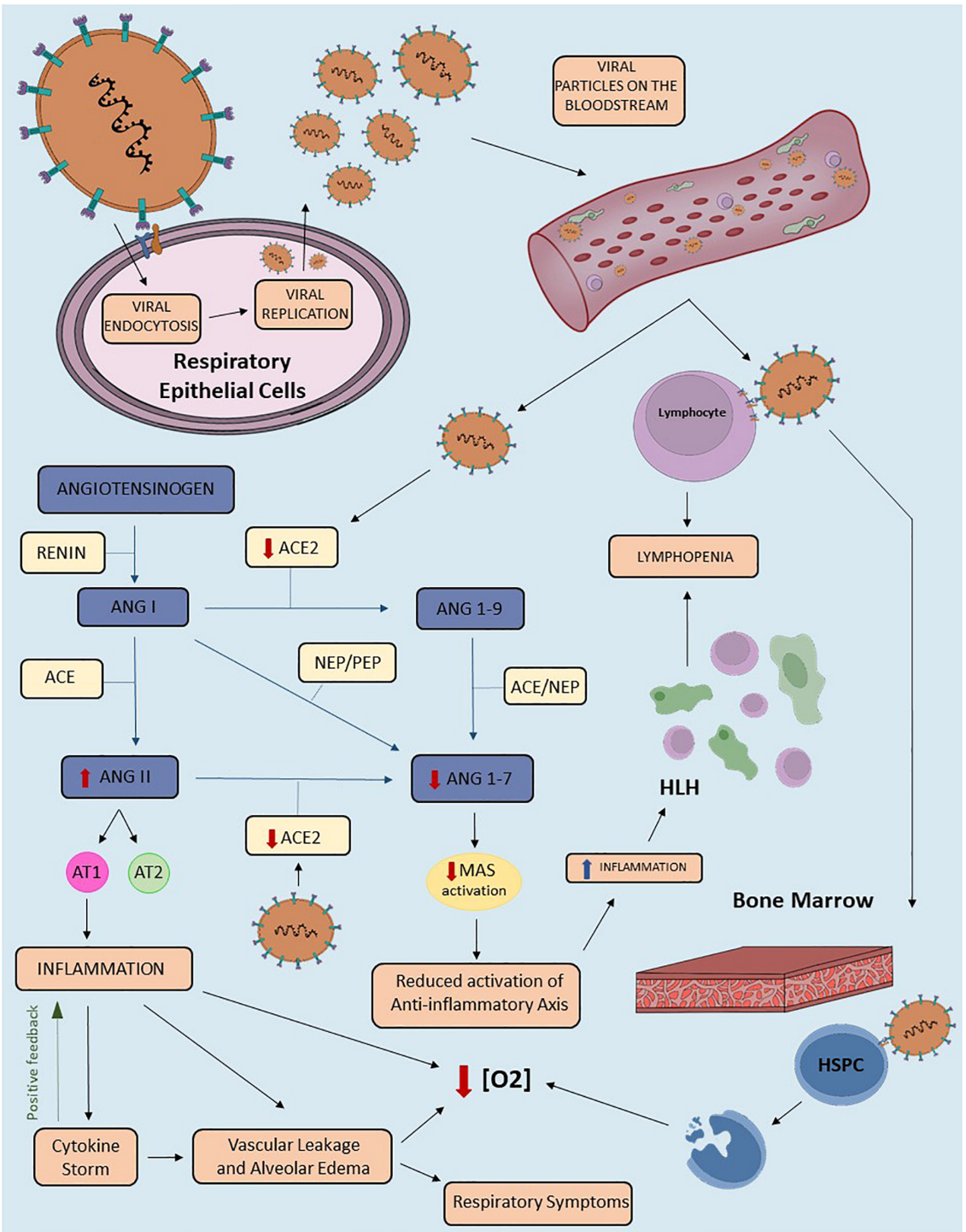


FIGURE 2 | The proposed role of the Renin-Angiotensin System in the pathophysiology of COVID-19. Schematic representation of COVID-19 pathophysiology related to the Renin-Angiotensin System (RAS) imbalance. This figure highlights the downregulation of transmembrane Angiotensin-converting enzyme 2 (ACE2) in SARS-CoV-2 infection. The virus enters the host cell after binding to TMPRSS2 and transmembrane ACE2. Viral replication and release from lung cells to the bloodstream enhance viremia, besides diminishing circulating and transmembrane ACE2 levels. The reduction of ACE2 availability results in RAS imbalance due to downregulation of the alternative axis. Consequently, we have an increase in Angiotensin II (Ang II) and decrease in Angiotensin-(1-7) [Ang-(1-7)] levels. The binding of Ang II to the Angiotensin II type 1 (AT1) receptor triggers inflammatory response, including vascular leakage and alveolar edema, both of which can be amplified by Cytokine Storm Syndrome (CSS). This mechanism may contribute to several clinical presentations of COVID-19, including respiratory signs and symptoms. In addition, the downregulation of the ACE2/Ang-(1-7)/Mas receptor axis reduces the anti-inflammatory effects of the alternative RAS axis. Due to ACE2 expression in

(Continued)

FIGURE 2 | Continued
mature lymphocytes, SARS-CoV-2 may result in lymphopenia. This finding can also be triggered by hemophagocytic lymphohistiocytosis (HLH) due to intense tissue inflammation. In addition, the invasion of the bone marrow by the virus, specifically of the hematopoietic stem/progenitor cells (HSPC), leads to apoptosis and consequent reduction of oxygen saturation levels. Other mechanisms that may contribute to lower saturation include vascular leakage, alveolar edema and inflammation. ACE2, Angiotensin Converting Enzyme 2; TMPRSS2, Transmembrane protease serine 2; RAS, Renin-Angiotensin-System; ANGII, Angiotensin II; ANG(1-7), Angiotensin (1-7); HLH, Hemophagocytic Lymphohistiocytosis; HSPC, Hematopoietic stem/progenitor cell; [O2], Oxygen concentration; ACE, Angiotensin Converting Enzyme; ANG(1-9), Angiotensin (1-9); ANGI, Angiotensin I; PEP, prolyl-endopeptidase; NEP, neutral-endopeptidase.

be responsible for the high mortality rates in COVID-19 patients that undergo mechanical ventilation (MV), as up to 80% of patients who required MV evolve to death (Richardson et al., 2020). The classical axis exacerbation possibly promotes endothelial dysfunction directly by Ang II effects and indirectly through immune system activation and hypoxia (Hu, 2020; Sardu et al., 2020). In addition, Ang II may act locally in the lungs' endothelium enhancing ROS production and reducing NO release (Forrester et al., 2018). All these pathophysiological mechanisms might result in vascular leakage and alveolar edema, causing hypoxia and dyspnea (Channappanavar and Perlman, 2017; Leiva-Juarez et al., 2018). In addition to a probable hepatocytes invasion by SARS-CoV-2, an exaggerated inflammatory response might lead to liver injury (Zhang C. et al., 2020). Elevated D-dimer levels, prothrombin time and International Normalized Ratio (INR) and reduced activated partial thromboplastin time (aPTT) are some laboratory findings that might be related to endothelium dysfunction and liver injury, which, in turn, increase the risk for thrombotic and thromboembolic events (Driggin et al., 2020; Huang et al., 2020; Tang et al., 2020; Wu et al., 2020).

There is an important gap between the second and third phases. Immune response and inflammation start right at the beginning of COVID-19, but, as the disease progresses, these mechanisms increase in intensity. SARS-CoV-2 binding to alveolar epithelial cells makes possible for the virus to activate innate and adaptive immune systems, leading to the release of several cytokines, including Interleukin (IL)-6. Previous studies demonstrated that some viral products (as the human immunodeficiency virus TAT protein transactivator) are able to enhance the DNA-binding activity of nuclear factor κB (NF-κB) and nuclear factor IL-6 (NF-IL-6), resulting in increased IL-6 mRNA transcription (Tisoncik et al., 2012; Tanaka et al., 2014). The same mechanism may also occur in COVID-19. In addition, due to the action of pro-inflammatory factors, vascular permeability increases and a large amount of fluid and blood cells get into the alveoli, resulting in dyspnea and respiratory failure (Channappanavar and Perlman, 2017; Leiva-Juarez et al., 2018). The IL-6 is usually synthesized locally on the lesion in the acute stage of inflammation, mediating pleiotropic effects on immune response and hematopoiesis (Tanaka et al., 2014). This cytokine also promotes specific differentiation of naïve CD4+ T cells and is indispensable for T-helper 17 (Th17) differentiation from naïve CD4+ T cells, being related to the disruption of immunological tolerance, auto-immune and chronic inflammatory diseases (Kimura and Kishimoto, 2010; Tanaka et al., 2014). IL-6 moves to the liver through the bloodstream and rapidly induces the release of several acute phase proteins, including C-reactive Protein (CRP) and fibrinogen (Tanaka et al., 2014). On the other hand, IL-6 reduces the production of fibronectin, albumin, and transferrin.

Third phase: this last stage is marked by a systemic hyper-inflammatory state named Cytokine Storm Syndrome (CSS) (Mehta et al., 2020). CSS seems to be responsible for worse clinical outcomes and represents an important maker of disease severity (Huang et al., 2020). Indeed, the association between ACE2 downregulation, exacerbation of the ACE/Ang II/AT1 axis and the release of several pro-inflammatory cytokines, including IL-1, IL-6, IL-8, and TNF-α, is well established in literature (Rodrigues Prestes et al., 2017). This finding might be enhanced by the activation of innate and adaptive immune systems triggered by the viral infection itself, which increases the activity of nuclear factors and mRNA transcription of interleukins. In addition, COVID-19 severity is associated with higher levels of IL-6, IL-2R, IL-10, and TNF-α, as well as lower CD4+ and CD8+ T cells (Pedersen and Ho, 2020). Furthermore, SARS-CoV-2 infection could trigger both primary and secondary HLH, an unusual syndrome characterized by fever, splenomegaly, jaundice and the histopathologic finding of hemophagocytosis in bone marrow and other tissues (Fisman, 2000). However, the mechanism by which viruses contribute to HLH development is not fully established. On this wise, previous studies analyzed the association between DNA viruses, the *Herpesviridae* family for instance, and the HLH, mainly because they are potent modulators of the immune response (Brisse et al., 2017). Less frequently, though, cases of HLH arise in RNA virus infections, including Influenza virus and DENV, among others (George, 2014).

Another important issue to be considered is the gut dysbiosis and its potential link to disease progression in COVID-19 (Aktas and Aslim, 2020; Dhar and Mohanty, 2020; Zuo et al., 2020). The detection of SARS-CoV-2 RNA in the stool of some patients and diarrhea in others point to a link between the lung and the intestine. Despite no fecal-oral transmission being reported up to this date, it's possible that asymptomatic children and adults may shed infectious virus particles in the stool, leading to infection of others (Dhar and Mohanty, 2020). Gut microbiota diversity and the presence of beneficial microorganisms in the gut may play an important role in determining the course of this disease (Aktas and Aslim, 2020; Dhar and Mohanty, 2020; Zuo et al., 2020). Interestingly, gut dysbiosis is present in several risk groups for COVID-19 as well, including the elderly, immune-compromised and diabetic patients (Dhar and Mohanty, 2020). In addition, ACE2 is highly expressed in the luminal surface of the gastrointestinal tract (Nehme et al., 2015), allowing the gastrointestinal tract to be colonized by SARS-CoV-2. This might explain why patients with COVID-19 exhibit gastrointestinal

discomfort and diarrhea. The ACE2 loss in the intestine is also related to the hyperactivation of classical RAS axis and the role of gut-lung axis in COVID-19 (Aktas and Aslim, 2020). SARS-CoV-2 infection may lead to degeneration of the gut blood barrier, driving to systemic propagation of bacteria and endotoxins, resulting in a septic shock. In this regard, a pilot study, including 15 patients with COVID-19, found persistent alterations in the fecal microbiome during the time of hospitalization (Zuo et al., 2020). Furthermore, fecal microbiota alterations were associated with fecal levels of SARS-CoV-2 and COVID-19 severity (Zuo et al., 2020). Additional studies are necessary to address the potential role of probiotics in COVID-19.

Risk Groups

Risk groups are subsets of the population that may probably evolve with the worst prognosis once ill, requiring special attention and more precaution warnings. For COVID-19, some well-established diseases and conditions are considered risk factors: diabetes, hypertension, chronic respiratory diseases, cardiovascular diseases, chronic kidney diseases, and cancer (Nikpouraghdam et al., 2020). Moreover, age and gender differences also seem to outline prognosis variety. Two other non-medical conditions require further investigation. The first one is pregnancy, due to the possibility of vertical transmission of SARS-CoV-2 to the fetus (Simões e Silva and Leal, 2020). The second one is related to the health workers, who are highly exposed to infected people. This exposure may predict a higher viral charge when infection is installed, which could result in higher disease severity. Epidemiological data further support RAS as a protagonist player in COVID-19 pathophysiology. In this regard, we present these risk groups establishing possible connections with RAS unbalanced.

Age groups: the most affected population is 50–60 years old (Chen et al., 2020) and epidemiological reports suggest a positive association between severity and aging. Patients with a severe form of disease were significantly older than the rest of the patients enrolled in a study conducted in China (Feng et al., 2020). In addition, the same study showed significantly lower survival rates in patients older than 75 years old, in comparison to younger patients (Feng et al., 2020). This age distribution of COVID-19 prognosis is not only related to the prevalence of preexistent comorbidities, but to the lifespan physiological oscillation of RAS molecules (Chen et al., 2020). In the aging process, there is a decrease in the estrogen/testosterone ratio, which promotes an increase in plasma renin activity, modifying the RAS axes equilibrium (Colafella et al., 2016). Furthermore, children and young adults have higher ACE2 reservoirs than elderly (Zhang P. et al., 2020). Thus, ACE/Ang II/AT1R is upregulated, an essential characteristic for COVID-19 bad evolution. This physiological mechanism is proven by the lower vascular and renal AT2 receptor expression, the raised AT1R expression and the enhanced pressor responsiveness to Ang II in female animal models, as well as the increased sensitivity to Ang II with aging male animal models (Colafella et al., 2016).

Hypertension and diabetes: epidemiological studies also showed worse outcome in patients with COVID-19 and associated comorbidities, including arterial hypertension and diabetes mellitus (Gheblawi et al., 2020; Vaduganathan et al., 2020). Indeed, these conditions are closely related to an exaggerated activation of ACE/Ang II/AT1R axis (Rein and Bader, 2017; Nistor et al., 2018; Schiffrin et al., 2020; Zheng et al., 2020). In diabetes, Ang II have been described to exert several deleterious effects, including increase in insulin resistance, endothelial damage and deterioration of renal function (Simões e Silva et al., 2017). Similarly, arterial hypertension courses with an inflammatory state, which includes higher levels of Ang II, chemokines and cytokines, including IL-6 and TNF-α (De Miguel et al., 2015). Therefore, a previous history of RAS imbalance favors the inflammatory state proposed to be responsible for disease severity in COVID-19. On the other hand, once again ACE2 expression might be crucial in prognosis: because several diabetic and/or hypertensive patients take RAS inhibitors to manage the classical axis upregulation.

RAS RELATED MEDICATIONS

The exposed mechanisms about COVID-19 pathophysiology explain the use of RAS-related medications in ongoing clinical trials. In this regard, RAS blocker therapy has been largely discussed on its beneficial or harmful effects. Considering that RAS inhibitors, like angiotensin-converting enzyme inhibitors (ACEi) and angiotensin II type 1 receptor antagonists (ARB), are first-line treatments for hypertension and diabetic nephropathy, some clinical trials aim to investigate whether RAS blockade therapy should be continued or not. Concerns about ACEi therapy are based on the proposition that these medications blunt the conversion of Ang I to Ang II. In addition, ACEi may also increase ACE2 expression and by doing so might favor SARS-CoV-2 binding to ACE2, the receptor for the virus (Fosbol et al., 2020; Perico et al., 2020). In this sense, the Irish CORONACION study (NCT04330300) enrolled 2414 patients with primarily hypertension to evaluate the association between RAS blocker administration and poorer prognosis. Similarly, French ACORES-2 trial (NCT04329195) separated 554 participants in two groups, one continuing RAS blocker therapy and one discontinuing it. Experimental findings, however, show inconclusive data regarding the effect of ACEi upon tissue levels of ACE2 (Bean et al., 2020). The risk of abrupt withdrawal of ACEi and ARB for patients chronically treated with these medications must be taken into account as well. In this regard, a retrospective cohort study using data from Danish national administrative registries concluded that prior use of ACEI/ARBs was not significantly associated with COVID-19 diagnosis among patients with hypertension or with mortality or severe disease among patients diagnosed as having COVID-19 (Fosbol et al., 2020). Therefore, several research groups advocate for treatment continuation in SARS-CoV-2 patients (AlGhatrif et al., 2020; Fosbol et al., 2020).

Despite the concerns about ARB medication by some researchers, other studies propose a potential therapeutic effect of these medications in COVID-19 (Vaduganathan et al., 2020). The general idea is based on the likely enhancement of ACE2 expression following chronic administration of ARB

(Rodrigues Prestes et al., 2017). Although the precise mechanisms beyond this upregulation of ACE2 require further characterization, experimental studies on heart, lung, and kidney tissues support this assumption (Rodrigues Prestes et al., 2017). The consequent upregulation of the RAS alternative arm might seem deleterious at first glance, since circulating ACE2 is directly derived from membrane-bound ACE2, minus the anchoring proteins. However, the further protective effects of the ACE2/Ang-(1-7)/MasR axis in the lungs have been considered beneficial in the final balance of RAS blocker therapy, as previously discussed in this article. Blocking the ACE/Ang II/AT1 axis, in this sense, diminishes Ang II lung injury due to its binding to the AT1 receptor and implies upregulation of the alternative arm, as raised ACE2 levels favors the conversion of Ang II into Ang-(1-7) (Rodrigues Prestes et al., 2017). **Figure 2** highlights the main effects of Ang-(1-7) binding to the Mas receptor. Additionally, the antagonism of the AT1R by ARAs could enhance the Ang II binding to the AT2 receptor, upregulated under chronic ARA administration, which may also exert protective effects in lung tissue (Vaduganathan et al., 2020). Several clinical trials aim to analyze the clinical outcomes of Losartan administration in patients positive for COVID-19. Two robust American trials aim to analyze the effect of a 7-day administration of Losartan 50 mg in comparison to placebo in patients positive to COVID-19 (NCT04312009, NCT04311177).

A different approach consists on the direct enhancement of the RAS alternative axis by the administration of Ang-(1-7) and Recombinant Human ACE2 (RhACE2). Belgic ATCO Trial will evaluate the results of Ang-(1-7) infusion in comparison to placebo in 60 participants positive for COVID-19 (NCT04332666). The proposed mechanism is based on the tentative of counter-regulating the exacerbation of the RAS classical arm. The administration of RhACE2, on its turn, might have two favorable effects: (1) the upregulation of the ACE2/Ang-(1-7)/MasR axis leading to and its beneficial actions and (2) the functionally neutralization of SARS-CoV-2 in the bloodstream. The second mechanism is possible due to the lacking of the membrane-anchoring domain in RhACE2. Therefore, it would not allow viral entrance into the host cell, but it is rather capable of antagonizing SARS-CoV-2 and preventing its endocytosis (Gheblawi et al., 2020). The binding of the virus to rhACE2 may also stimulate the immune system to counteract SARS-CoV-2 (Gheblawi et al., 2020). Although being promising therapeutic alternatives to COVID-19, treatment with these peptides face some major challenges, including the short half-life *in vivo*, low stability, high manufacturing cost and rapid degradation in the gastrointestinal tract when administered orally, meaning the need of a continuous intravenous infusion (Shenoy et al., 2015). In this sense, the one trial proposing RhACE2 administration to treat COVID-19 still lacks the Center of Drug Evaluation approval (NCT04287686). Further pharmacological investigation on these peptides could represent a brand new therapeutic perspective for COVID-19, as well as for other diseases related to RAS imbalance.

CONCLUDING REMARKS

Coronavirus Disease 2019 pathogenesis and pathophysiology are far from being fully elucidated. The first studies considered different stages of the diseases separately and without well-established mechanisms. In this context, we do believe that RAS imbalance may exert an important role in COVID-19. The shift of RAS equilibrium toward the classical axis, ACE/Ang II/AT1R, in parallel with downregulation of the alternative axis, ACE2/Ang-(1-7)/MasR, may contribute to the plethora of clinical manifestations of COVID-19 and its severity. In this regard, we defend that the novel therapeutic and preventive strategies take into account the importance of restoring RAS equilibrium.

AUTHOR CONTRIBUTIONS

LC and LP made the literature revision and selection of main manuscripts and wrote the first draft of the review. VP, TM, and VR defined the topics of this review and helped in writing the first draft. TM and LC made the figures. AS and KL conceptualized the study, made general supervision, and revised the manuscript. AS submitted the final version of the manuscript, which is approved by all authors.

FUNDING

This work was partially supported by the Brazilian National Council of Research Development (CNPq – Grant # 302153/2019-5), Coordination for the Improvement of Higher Education Personnel (CAPES), and Foundation of Research of Minas Gerais (FAPEMIG).

REFERENCES

Aktas, B., and Aslim, B. (2020). Gut-lung axis and dysbiosis in COVID-19. *Turkish J. Biol.* 44, 265–272. doi: 10.3906/biy-2005-102

AlGhatrif, M., Cingolani, O., and Lakatta, E. G. (2020). The dilemma of Coronavirus Disease 2019, aging, and cardiovascular disease: insights from cardiovascular aging science. *JAMA Cardiol.* 5, 747–748. doi: 10.1001/jamacardio.2020.1329

Ardaillou, R. (1998). Angiotensin II-complexities beyond AT1 and AT2 receptors. *Nephrol. Dial. Transplant.* 13, 2988–2990. doi: 10.1093/ndt/13.12.2988

Bean, D., Kraljevic, Z., Searle, T., Bendayan, R., Pickles, A., Folarin, A., et al. (2020). Treatment with ACE-inhibitors is associated with less severe disease with SARS-Covid-19 infection in a multi-site UK acute Hospital Trust. *medRxiv* [Preprint]. doi: 10.13140/RG.2.2.34883.14889/1

Bosch, B. J., Bartelink, W., and Rottier, P. J. (2008). Cathepsin L functionally cleaves the severe acute respiratory syndrome coronavirus class I fusion protein upstream of rather than adjacent to the fusion peptide. *J. Virol.* 82, 8887–8890. doi: 10.1128/jvi.00415-08

Bright, N. A., Davis, L. J., and Luzio, J. P. (2016). Endolysosomes are the principal intracellular sites of acid hydrolase activity. *Curr. Biol.* 26, 2233–2245. doi: 10.1016/j.cub.2016.06.046

Brisse, E., Wouters, C. H., Andrei, G., and Matthys, P. (2017). How viruses contribute to the pathogenesis of hemophagocytic lymphohistiocytosis. *Front. Immunol.* 8:1102. doi: 10.3389/fimmu.2017.01102

Channappanavar, R., and Perlman, S. (2017). Pathogenic human coronavirus infections: causes and consequences of cytokine storm and immunopathology. *Semin. Immunopathol.* 39, 529–539. doi: 10.1007/s00281-017-0629-x

Chen, Z., Mi, L., Xu, J., Yu, J., Wang, X., Jiang, J., et al. (2005). Function of HAb18G/CD147 in invasion of host cells by severe acute respiratory syndrome coronavirus. *J. Infect. Dis.* 191, 755–760. doi: 10.1086/427811

Chen, Z. M., Fu, J. F., Shu, Q., Chen, Y. H., Hua, C. Z., Li, F. B., et al. (2020). Diagnosis and treatment recommendations for pediatric respiratory infection caused by the 2019 novel coronavirus. *World J. Pediatr.* 16, 240–246. doi: 10.1007/s12519-020-00345-5

Colafella, K. M., Hilliard, L. M., and Denton, K. M. (2016). Epochs in the depressor/pressor balance of the renin-angiotensin system. *Clin. Sci.* 130, 761–771. doi: 10.1042/cs20150939

Coronaviridae Study Group of the International Committee on Taxonomy of Viruses (2020). The species Severe acute respiratory syndrome-related coronavirus: classifying 2019-nCoV and naming it SARS-CoV-2. *Nat. Microbiol.* 5, 536–544. doi: 10.1038/s41564-020-0695-z

Coutard, B., Valle, C., de Lamballerie, X., Canard, B., Seidah, N., and Decroly, E. (2020). The spike glycoprotein of the new coronavirus 2019-nCoV contains a furin-like cleavage site absent in CoV of the same clade. *Antivir. Res.* 176:104742. doi: 10.1016/j.antiviral.2020.104742

D'Ardes, D., Boccatonda, A., Rossi, I., Guagnano, M. T., Santilli, F., Cipollone, F., et al. (2020). COVID-19 and RAS: unravelling an unclear relationship. *Int. J. Mol. Sci.* 21:3003. doi: 10.3390/ijms21083003

De Miguel, C., Rudemiller, N., Abais, J., and Mattson, D. (2015). Inflammation and hypertension: new understandings and potential therapeutic targets. *Curr. Hypertens Rep.* 17:507.

Dhar, D., and Mohanty, A. (2020). Gut microbiota and Covid-19 - possible link and implications. *Virus Res.* 285:198018. doi: 10.1016/j.virusres.2020.198018

Donoghue, M., Hsieh, F., Baronas, E., Godbout, K., Gosselin, M., Stagliano, N., et al. (2000). A novel angiotensin-converting enzyme-related carboxypeptidase (ACE2) converts angiotensin I to angiotensin 1-9. *Circ. Res.* 87, E1–E9.

Driggin, E., Madhavan, M. V., Bikdeli, B., Chuich, T., Laracy, J., Bondi-Zoccai, G., et al. (2020). Cardiovascular considerations for patients, health care workers, and health systems during the Coronavirus Disease 2019 (COVID-19) Pandemic. *J. Am. Coll Cardiol.* 75, 2352–2371.

Feng, Y., Ling, Y., Bai, T., Xie, Y., Huang, J., Li, J., et al. (2020). COVID-19 with different severity: a multi-center study of clinical features. *Am. J. Respir. Crit. Care Med.* 201, 1380–1388.

Fisman, D. N. (2000). Hemophagocytic syndromes and infection. *Emerg. Infect. Dis.* 6, 601–608. doi: 10.3201/eid0606.000608

Forrester, S. J., Booz, G. W., Sigmund, C. D., Coffman, T. M., Kawai, T., Rizzo, V., et al. (2018). Angiotensin II signal transduction: an update on mechanisms of physiology and pathophysiology. *Physiol. Rev.* 98, 1627–1738. doi: 10.1152/physrev.00038.2017

Fosbol, E., Butt, J. H., Ostergaaard, L., Andersson, C., Selmer, C., Kragholm, K., et al. (2020). Association of angiotensin-converting enzyme inhibitor or angiotensin receptor blocker use with COVID-19 diagnosis and mortality. *JAMA* 324, 168–177. doi: 10.1001/jama.2020.11301

Gattinoni, L., Chiumello, D., Caironi, P., Busana, M., Romitti, F., Brazzi, L., et al. (2020a). COVID-19 pneumonia: different respiratory treatments for different phenotypes? *Intensive Care Med.* 46, 1099–1102. doi: 10.1007/s00134-020-06033-2

Gattinoni, L., Coppola, S., Cressoni, M., Busana, M., Rossi, S., and Chiumello, D. (2020b). Covid-19 does not lead to a typical acute respiratory distress syndrome. *Am. J. Respir. Crit. Care Med.* 201, 1299–1300.

George, M. R. (2014). Hemophagocytic lymphohistiocytosis: review of etiologies and management. *J. Blood Med.* 5, 69–86. doi: 10.2147/jbm.s46255

Gheblawi, M., Wang, K., Viveiros, A., Nguyen, Q., Zhong, J. C., Turner, A. J., et al. (2020). Angiotensin converting enzyme 2: SARS-CoV-2 receptor and regulator of the renin-angiotensin system. *Circ. Res.* 126, 1456–1474. doi: 10.1161/circresaha.120.317015

Glowacka, I., Bertram, S., Herzog, P., Pfefferle, S., Steffen, I., Muench, M. O., et al. (2010). Differential downregulation of ACE2 by the spike proteins of severe acute respiratory syndrome coronavirus and human coronavirus NL63. *J. Virol.* 84, 1198–1205. doi: 10.1128/jvi.01248-09

Gordon, D., Jang, G., Bouhaddou, M., Xu, J., Obernier, K., White, K., et al. (2020). A SARS-CoV-2 protein interaction map reveals targets for drug repurposing. *Nature* 583, 459–468. doi: 10.1038/s41586-020-2286-9

Gu, H., Xie, Z., Li, T., Zhang, S., Lai, C., Zhu, P., et al. (2016). Angiotensin-converting enzyme 2 inhibits lung injury induced by respiratory syncytial virus. *Sci. Rep.* 6:19840.

Guzzi, P., Mercatelli, D., Ceraolo, C., and Giorgi, F. (2020). Master regulator analysis of the SARS-CoV-2/Human interactome. *J. Clin. Med.* 9:982. doi: 10.3390/jcm9040982

Hoffmann, M., Kleine-Weber, H., Schroeder, S., Kruger, N., Herrler, T., Erichsen, S., et al. (2020). SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor. *Cell* 181, 271.e8–280.e8.

Hu, S. (2020). Comment on Organ-protective effect of angiotensin-converting enzyme 2 and its effect on the prognosis of COVID-19. *J. Med. Virol.* 92, 1425–1426. doi: 10.1002/jmv.25937

Huang, C., Wang, Y., Li, X., Ren, L., Zhao, J., Hu, Y., et al. (2020). Clinical features of patients infected with 2019 novel coronavirus in Wuhan. China. *Lancet* 395, 497–506.

Imai, Y., Kuba, K., Rao, S., Huan, Y., Guo, F., Guan, B., et al. (2005). Angiotensin-converting enzyme 2 protects from severe acute lung failure. *Nature* 436, 112–116. doi: 10.1038/nature03712

Jia, H. (2016). Pulmonary angiotensin-converting enzyme 2 (ACE2) and inflammatory lung disease. *Shock* 46, 239–248. doi: 10.1097/shk.0000000000000633

Joshi, S., Wollenzien, H., Leclerc, E., and Jarajapu, Y. P. (2019). Hypoxic regulation of angiotensin-converting enzyme 2 and Mas receptor in human CD34(+) cells. *J. Cell Physiol.* 234, 20420–20431. doi: 10.1002/jcp.28643

Kawase, M., Shirato, K., van der Hoek, L., Taguchi, F., and Matsuyama, S. (2012). Simultaneous treatment of human bronchial epithelial cells with serine and cysteine protease inhibitors prevents severe acute respiratory syndrome coronavirus entry. *J. Virol.* 86, 6537–6545. doi: 10.1128/jvi.00094-12

Kimura, A., and Kishimoto, T. (2010). IL-6: regulator of Treg/Th17 balance. *Eur. J. Immunol.* 40, 1830–1835. doi: 10.1002/eji.201040391

Kuba, K., Imai, Y., and Penninger, J. M. (2006). Angiotensin-converting enzyme 2 in lung diseases. *Curr. Opin. Pharmacol.* 6, 271–276.

Lanza, K., Perez, L. G., Costa, L. B., Cordeiro, T. M., Palmeira, V. A., and Ribeiro, V. T. (2020). Covid-19: the renin–angiotensin system imbalance hypothesis. *Clin. Sci.* 134, 1259–1264. doi: 10.1042/cs20200492

Lechien, J. R., Chiesa-Estomba, C. M., De Siati, D. R., Horoi, M., Le Bon, S. D., Rodriguez, A., et al. (2020). Olfactory and gustatory dysfunctions as a clinical presentation of mild-to-moderate forms of the coronavirus disease (COVID-19): a multicenter European study. *Eur. Arch. Otorhinolaryngol.* 277, 2251–2261. doi: 10.1007/s00405-020-05965-1

Lee, N., Hui, D., Wu, A., Chan, P., Cameron, P., Joynt, G. M., et al. (2003). A major outbreak of severe acute respiratory syndrome in Hong Kong. *N. Engl. J. Med.* 348, 1986–1994.

Leiva-Juarez, M. M., Kolls, J. K., and Evans, S. E. (2018). Lung epithelial cells: therapeutically inducible effectors of antimicrobial defense. *Mucosal Immunol.* 11, 21–34. doi: 10.1038/mi.2017.71

Letko, M., Marzi, A., and Munster, V. (2020). Functional assessment of cell entry and receptor usage for SARS-CoV-2 and other lineage B betacoronaviruses. *Nat. Microbiol.* 5, 562–569. doi: 10.1038/s41564-020-0688-y

Li, M. Y., Li, L., Zhang, Y., and Wang, X. S. (2020). Expression of the SARS-CoV-2 cell receptor gene ACE2 in a wide variety of human tissues. *Infect. Dis. Poverty* 9:45.

Li, Q., Guan, X., Wu, P., Wang, X., Zhou, L., Tong, Y., et al. (2020). Early transmission dynamics in Wuhan, China, of Novel Coronavirus-infected pneumonia. *N. Engl. J. Med.* 382, 1199–1207.

Li, W., Hulswit, R. J. G., Widjaja, I., Raj, V. S., McBride, R., Peng, W., et al. (2017). Identification of sialic acid-binding function for the middle east respiratory syndrome coronavirus spike glycoprotein. *Proc. Natl. Acad. Sci. U.S.A.* 114, E8508–E8517.

Liu, Z., Xiao, X., Wei, X., Li, J., Yang, J., Tan, H., et al. (2020). Composition and divergence of coronavirus spike proteins and host ACE2 receptors predict potential intermediate hosts of SARS-CoV-2. *J. Med. Virol.* 92, 595–601. doi: 10.1002/jmv.25726

Lu, R., Zhao, X., Li, J., Niu, P., Yang, B., Wu, H., et al. (2020). Genomic characterisation and epidemiology of 2019 novel coronavirus: implications for

virus origins and receptor binding. *Lancet* 395, 565–574. doi: 10.1016/s0140-6736(20)30251-8

Magalhaes, G. S., Barroso, L. C., Reis, A. C., Rodrigues-Machado, M. G., Gregório, J. F., Motta-Santos, D., et al. (2018). Angiotensin-(1–7) promotes resolution of eosinophilic inflammation in an experimental model of asthma. *Front. Immunol.* 9:58. doi: 10.3389/fimmu.2018.00058

Maginnis, M. S. (2018). Virus-receptor interactions: the key to cellular invasion. *J. Mol. Biol.* 430, 2590–2611. doi: 10.1016/j.jmb.2018.06.024

Mehta, P., McAuley, D. F., Brown, M., Sanchez, E., Tattersall, R. S., and Manson, J. J. (2020). COVID-19: consider cytokine storm syndromes and immunosuppression. *Lancet* 395, 1033–1034. doi: 10.1016/s0140-6736(20)30628-0

Millet, J. K., and Whittaker, G. R. (2015). Host cell proteases: critical determinants of coronavirus tropism and pathogenesis. *Virus Res.* 202, 120–134. doi: 10.1016/j.virusres.2014.11.021

Musa, S. (2020). Hepatic and gastrointestinal involvement in coronavirus disease 2019 (COVID-19): what do we know till now? *Arab. J. Gastroenterol.* 21, 3–8. doi: 10.1016/j.ajg.2020.03.002

Naslavsky, N., and Caplan, S. (2018). The enigmatic endosome - sorting the ins and outs of endocytic trafficking. *J. Cell Sci.* 131:jcs216499. doi: 10.1242/jcs.216499

Nehme, A., Cerutti, C., Dhaouadi, N., Gustin, M. P., Courand, P. Y., Zibara, K., et al. (2015). Atlas of tissue renin-angiotensin-aldosterone system in human: a transcriptomic meta-analysis. *Sci. Rep.* 5:10035.

Nikpouraghdam, M., Jalali Farahani, A., Alishiri, G., Heydari, S., Ebrahimnia, M., Samadinia, H., et al. (2020). Epidemiological characteristics of coronavirus disease 2019 (COVID-19) patients in IRAN: a single center study. *J. Clin. Virol.* 127:104378.

Nistor, I., De Sutter, J., Drechsler, C., Goldsmith, D., Soler, M. J., Tomson, C., et al. (2018). Effect of renin-angiotensin-aldosterone system blockade in adults with diabetes mellitus and advanced chronic kidney disease not on dialysis: a systematic review and meta-analysis. *Nephrol. Dial. Transplant* 33, 12–22. doi: 10.1093/ndt/gfx072

Ou, X., Liu, Y., Lei, X., Li, P., Mi, D., Ren, L., et al. (2020). Characterization of spike glycoprotein of SARS-CoV-2 on virus entry and its immune cross-reactivity with SARS-CoV. *Nat. Commun.* 11:1620.

Patel, S., Rauf, A., Khan, H., and Abu-Izneid, T. (2017). Renin-angiotensin-aldosterone (RAAS): the ubiquitous system for homeostasis and pathologies. *Biomed. Pharmacother.* 94, 317–325. doi: 10.1016/j.biopha.2017.07.091

Pedersen, S. F., and Ho, Y. C. (2020). SARS-CoV-2: a storm is raging. *J. Clin. Invest.* 130, 2202–2205. doi: 10.1172/jci137647

Perico, L., Benigni, A., and Remuzzi, G. (2020). Should COVID-19 Concern Nephrologists? why and to what extent? The emerging impasse of angiotensin blockade. *Nephron* 144, 251–252. doi: 10.1159/000507786

Pushkarsky, T., Zybarth, G., Dubrovsky, L., Yurchenko, V., Tang, H., Guo, H., et al. (2001). CD147 facilitates HIV-1 infection by interacting with virus-associated cyclophilin A. *Proc. Natl. Acad. Sci. U.S.A.* 98, 6360–6365. doi: 10.1073/pnas.111583198

Qian, Z., Dominguez, S. R., and Holmes, K. V. (2013). Role of the spike glycoprotein of human middle east respiratory syndrome coronavirus (MERS-CoV) in virus entry and syncytia formation. *PLoS One* 8:e76469. doi: 10.1371/journal.pone.0076469

Rachakonda, P. S., Veit, M., Korte, T., Ludwig, K., Bottcher, C., Huang, Q., et al. (2007). The relevance of salt bridges for the stability of the influenza virus hemagglutinin. *FASEB J.* 21, 995–1002. doi: 10.1096/fj.06-7052hyp

Radzikowska, U., Ding, M., Tan, G., Zhakparov, D., Peng, Y., Wawrzyniak, P., et al. (2020). Distribution of ACE2, CD147, CD26 and other SARS-CoV-2 associated molecules in tissues and immune cells in health and in asthma, COPD, obesity, hypertension, and COVID-19 risk factors. *Allergy* doi: 10.1111/all.14429 [Epub ahead of print].

Rein, J., and Bader, M. (2017). Renin-angiotensin system in diabetes. *Protein Pept. Lett.* 24, 833–840.

Richardson, S., Hirsch, J. S., Narasimhan, M., Crawford, J. M., McGinn, T., and Davidson, K. W. (2020). Presenting Characteristics, Comorbidities, and outcomes among 5700 patients hospitalized with COVID-19 in the New York City Area. *JAMA* 323, 2052–2059. doi: 10.1001/jama.2020.6775

Rodrigues Prestes, T. R. N., Rocha, P., Miranda, A. S., Teixeira, A. L., and Simoes, E. S. A. C. (2017). The anti-inflammatory potential of ACE2/Angiotensin-(1-7)/Mas Receptor Axis: evidence from basic and clinical research. *Curr. Drug Targets* 18, 1301–1313.

Rodriguez-Morales, A. J., Cardona-Ospina, J. A., Gutierrez-Ocampo, E., Villamizar-Pena, R., Holguin-Rivera, Y., and Escalera-Antezana, J. P. (2020). Clinical, laboratory and imaging features of COVID-19: a systematic review and meta-analysis. *Travel Med. Infect. Dis.* 34:101623.

Rothan, H. A., and Byrareddy, S. N. (2020). The epidemiology and pathogenesis of coronavirus disease (COVID-19) outbreak. *J. Autoimmun.* 109:102433. doi: 10.1016/j.jaut.2020.102433

Sagkan, R. I., and Akin-Bali, D. F. (2020). Structural variations and expression profiles of the SARS-CoV-2 host invasion genes in Lung cancer. *J. Med. Virol.* doi: 10.1002/jmv.26107 [Epub ahead of print].

Santos, R. A. S., Campagnole-Santos, M. J., and Andrade, S. P. (2000). Angiotensin-(1-7): an update. *Regul. Pept.* 91, 45–62.

Santos, R. A. S., Ferreira, A. J., Pinheiro, S. V., Sampaio, W. O., Touyz, R., and Campagnole-Santos, M. J. (2005). Angiotensin-(1-7) and its receptor as a potential targets for new cardiovascular drugs. *Expert Opin. Investig Drugs* 14, 1019–1031. doi: 10.1517/13543784.14.8.1019

Santos, R. A. S., Sampaio, W. O., Alzamora, A. C., Motta-Santos, D., Alenina, N., Bader, M., et al. (2018). The ACE2/Angiotensin-(1-7)/MAS Axis of the renin-angiotensin system: focus on angiotensin-(1-7). *Physiol. Rev.* 98, 505–553. doi: 10.1152/physrev.00023.2016

Santos, R. A. S., Santos, K., Brosnihan, B., Chappell, M. C., Pesquero, J., Chernicky, C. L., et al. (1988). Converting enzyme activity and angiotensin metabolismo in the dog brainstem. *Hypertension* 11, I153–1157.

Santos, R. A. S., Simoes e Silva, A. C., Maric, C., Silva, D. M., Machado, R. P., de Buhr, I., et al. (2003). Angiotensin-(1-7) is an endogenous ligand for the G protein-coupled receptor Mas. *Proc. Natl. Acad. Sci. U.S.A.* 100, 8258–8263.

Sardu, C., Gambardella, J., Morelli, M. B., Wang, X., Marfella, R., Santulli, G. et al. (2020). Is COVID-19 an endothelial disease? Clinical and basic evidence. *J. Clin. Med.* 9:1417. doi: 10.3390/jcm9051417

Schiavone, M. T., Santos, R. A. S., Brosnihan, K. B., Khosla, M. C., and Ferrario, C. M. (1988). Release of vasopressin from the rat hypothalamo-neurohypophysial system by angiotensin-(1-7) heptapeptide. *Proc. Natl. Acad. Sci. U.S.A.* 85, 4095–4098. doi: 10.1073/pnas.85.11.4095

Schiffrin, E. L., Flack, J. M., Ito, S., Muntner, P., and Webb, R. C. (2020). Hypertension and COVID-19. *Am. J. Hypertens* 33, 373–374.

Shang, J., Wan, Y., Luo, C., Ye, G., Geng, Q., Auerbach, A., et al. (2020a). Cell entry mechanisms of SARS-CoV-2. *Proc. Natl. Acad. Sci. U.S.A.* 117, 11727–11734. doi: 10.1073/pnas.2003138117

Shang, J., Ye, G., Shi, K., Wan, Y., Luo, C., Aihara, H., et al. (2020b). Structural basis of receptor recognition by SARS-CoV-2. *Nature* 581, 221–224. doi: 10.1038/s41586-020-2179-y

Shenoy, V., Ferreira, A. J., Katovich, M., and Raizada, M. K. (2015). "Angiotensin-converting enzyme 2/Angiotensin-(1-7)/Mas receptor axis: emerging pharmacological target for pulmonary diseases," in *The Protective Arm of The Renin Angiotensin System*, eds T. Unger, U. M. Steckelings, and R. A. S. Santos (Amsterdam: Elsevier), 269–274. doi: 10.1016/b978-0-12-801364-9.00038-9

Shulla, A., Heald-Sargent, T., Subramanya, G., Zhao, J., Perlman, S., and Gallagher, T. (2011). A transmembrane serine protease is linked to the severe acute respiratory syndrome coronavirus receptor and activates virus entry. *J. Virol.* 85, 873–882. doi: 10.1128/jvi.02062-10

Sigrist, C. J., Bridge, A., and Le Mercier, P. (2020). A potential role for integrins in host cell entry by SARS-CoV-2. *Antivir. Res.* 177:104759. doi: 10.1016/j.antiviral.2020.104759

Simmons, G., Gosalia, D. N., Rennekamp, A. J., Reeves, J. D., Diamond, S. L., and Bates, P. (2005). Inhibitors of cathepsin L prevent severe acute respiratory syndrome coronavirus entry. *Proc. Natl. Acad. Sci. U.S.A.* 102, 11876–11881. doi: 10.1073/pnas.0505577102

Simões e Silva, A., and Leal, C. (2020). Is SARS-CoV-2 vertically transmitted? *Front. Pediatr.* 8:276.

Simões e Silva, A., and Teixeira, M. (2016). ACE inhibition, ACE2 and angiotensin-(1-7) axis in kidney and cardiac inflammation and fibrosis. *Pharmacol. Res.* 107, 154–162. doi: 10.1016/j.phrs.2016.03.018

Simões e Silva, A. C., Ferreira, R. N., and Miranda, A. S. (2017). "The renin angiotensin system and diabetes," in *Mechanisms of Vascular Defects in Diabetes*

Mellitus, 1st Edn, eds C. C. Kartha, S. Ramachandran, and R. M. Pillai (Berlin: Springer), 575.

Simões e Silva, A. C., and Flynn, J. T. (2012). The renin-angiotensin-aldosterone system in 2011: role in hypertension and chronic kidney disease. *Pediatr. Nephrol.* 27, 1835–1845. doi: 10.1007/s00467-011-2002-y

Sodhi, C. P., Nguyen, J., Yamaguchi, Y., Werts, A. D., Lu, P., Ladd, M. R., et al. (2019). A Dynamic variation of pulmonary ACE2 Is required to modulate neutrophilic inflammation in response to *Pseudomonas aeruginosa* lung infection in mice. *J. Immunol.* 203, 3000–3012. doi: 10.4049/jimmunol.1900579

Srinivasan, S., Cui, H., Gao, Z., Liu, M., Lu, S., Mkandawire, W., et al. (2020). Structural genomics of SARS-CoV-2 indicates evolutionary conserved functional regions of viral proteins. *Viruses* 12:360. doi: 10.3390/v12040360

Stewart, P. L., and Nemerow, G. R. (2007). Cell integrins: commonly used receptors for diverse viral pathogens. *Trends Microbiol.* 15, 500–507. doi: 10.1016/j.tim.2007.10.001

Szabo, R., and Bugge, T. H. (2008). Type II transmembrane serine proteases in development and disease. *Int. J. Biochem. Cell Biol.* 40, 1297–1316. doi: 10.1016/j.biocel.2007.11.013

Szabo, R., and Bugge, T. H. (2011). Membrane-anchored serine proteases in vertebrate cell and developmental biology. *Annu. Rev. Cell Dev. Biol.* 27, 213–235. doi: 10.1146/annurev-cellbio-092910-154247

Tan, L., Wang, Q., Zhang, D., Ding, J., Huang, Q., Tang, Y. Q., et al. (2020). Lymphopenia predicts disease severity of COVID-19: a descriptive and predictive study. *Signal. Transduct. Target Ther.* 5:33.

Tanaka, T., Narazaki, M., and Kishimoto, T. (2014). IL-6 in inflammation, immunity, and disease. *Cold Spring Har.b Perspect. Biol.* 6:a016295.

Tang, N., Li, D., Wang, X., and Sun, Z. (2020). Abnormal coagulation parameters are associated with poor prognosis in patients with novel coronavirus pneumonia. *J. Thromb. Haemost.* 18, 844–847. doi: 10.1111/jth.14768

Tarnow, C., Engels, G., Arendt, A., Schwalm, F., Sediri, H., Preuss, A., et al. (2014). TMPRSS2 is a host factor that is essential for pneumotropism and pathogenicity of H7N9 influenza a virus in mice. *J. Virol.* 88, 4744–4751. doi: 10.1128/jvi.03799-13

Tipnis, S. R., Hooper, N. M., Hyde, R., Karran, E., Christie, G., and Turner, A. J. (2000). A human homolog of angiotensin-converting enzyme. Cloning and functional expression as a captopril-insensitive carboxypeptidase. *J. Biol. Chem.* 275, 33238–33243. doi: 10.1074/jbc.m002615200

Tisoncik, J. R., Korth, M. J., Simmons, C. P., Farrar, J., Martin, T. R., and Katze, M. G. (2012). Into the eye of the cytokine storm. *Microbiol. Mol. Biol. Rev.* 76, 16–32.

Turk, V., Stoka, V., Vasiljeva, O., Renko, M., Sun, T., Turk, B., et al. (2012). Cysteine cathepsins: from structure, function and regulation to new frontiers. *Biochim. Biophys. Acta* 1824, 68–88. doi: 10.1016/j.bbapap.2011.10.002

Tyrrell, D. A., and Bynoe, M. L. (1965). Cultivation of a novel type of common-cold virus in organ cultures. *Br. Med. J.* 1, 1467–1470. doi: 10.1136/bmj.1.5448.1467

Ulrich, H., and Pillat, M. M. (2020). CD147 as a target for COVID-19 treatment: suggested effects of azithromycin and stem cell engagement. *Stem Cell Rev. Rep.*16, 434–440. doi: 10.1007/s12015-020-09976-7

Vaduganathan, M., Vardeny, O., Michel, T., McMurray, J. J. V., Pfeffer, M. A., and Solomon, S. D. (2020). Renin-angiotensin-aldosterone system inhibitors in patients with Covid-19. *N. Engl. J. Med.* 382, 1653–1659.

Verdecchia, P., Cavallini, C., Spanevello, A., and Angeli, F. (2020). The pivotal link between ACE2 deficiency and SARS-CoV-2 infection. *Eur. J. Int. Med.* 76, 14–20. doi: 10.1016/j.ejim.2020.04.037

Walls, A. C., Park, Y. J., Tortorici, M. A., Wall, A., McGuire, A. T., and Veesler, D. (2020). Structure, function, and antigenicity of the SARS-CoV-2 spike glycoprotein. *Cell* 181, 281.e6–292.e6.

Wan, Y., Shang, J., Graham, R., Baric, R. S., and Li, F. (2020). Receptor Recognition by the novel coronavirus from wuhan: an analysis based on decade-long structural studies of SARS Coronavirus. *J. Virol.* 94:e00127-20. doi: 10.1128/JVI.00127-20

Wang, D., Hu, B., Hu, C., Zhu, F., Liu, X., Zhang, J., et al. (2020). Clinical characteristics of 138 Hospitalized Patients With 2019 Novel Coronavirus-Infected Pneumonia in Wuhan. China. *Jama* 323, 1061–1069. doi: 10.1001/jama.2020.1585

Wang, K., Chen, W., Zhou, Y.-S., Lian, J.-Q., Zhang, Z., Du, P., et al. (2020). SARS-CoV-2 invades host cells via a novel route: CD147-spike protein. *bioRxiv* [Preprint]. doi: 10.1101/2020.03.14.988345

Wang, Q., Zhang, Y., Wu, L., Niu, S., Song, C., Zhang, Z., et al. (2020). Structural and Functional Basis of SARS-CoV-2 Entry by Using Human ACE2. *Cell* 181, 894.e9–904.e9.

White, J. M., Delos, S. E., Brecher, M., and Schornberg, K. (2008). Structures and mechanisms of viral membrane fusion proteins: multiple variations on a common theme. *Crit. Rev. Biochem. Mol. Biol.* 43, 189–219. doi: 10.1080/10409230802058320

Wrapp, D., Wang, N., Corbett, K. S., Goldsmith, J. A., Hsieh, C. L., Abiona, O., et al. (2020). Cryo-EM structure of the 2019-nCoV spike in the prefusion conformation. *Science* 367, 1260–1263.

Wu, C., Chen, X., Cai, Y., Xia, J., Zhou, X., Xu, S., et al. (2020). Risk factors associated with acute respiratory distress syndrome and death in patients With Coronavirus Disease 2019 Pneumonia in Wuhan, China. *JAMA Int. Med.* 180, 1–11. doi: 10.1155/2019/2476845

Yamada, K., Iyer, S. N., Chappell, M. C., Ganten, D., and Ferrario, C. M. (1998). Converting enzyme determines plasma clearance of angiotensin-(1–7). *Hypertension* 32, 496–502. doi: 10.1161/01.hyp.32.3.496

Zhang, C., Shi, L., and Wang, F. S. (2020). Liver injury in COVID-19: management and challenges. *Lancet Gastroenterol. Hepatol.* 5, 428–430. doi: 10.1016/s2468-1253(20)30057-1

Zhang, P., Zhu, L., Cai, J., Lei, F., Qin, J. J., Xie, J., et al. (2020). Association of inpatient use of angiotensin converting enzyme inhibitors and angiotensin II receptor blockers with mortality among patients with Hypertension Hospitalized With COVID-19. *Circ. Res.* 126, 1671–1681.

Zhang, Y. Z., and Holmes, E. C. (2020). A genomic perspective on the origin and emergence of SARS-CoV-2. *Cell* 181, 223–227. doi: 10.1016/j.cell.2020.03.035

Zheng, Y. Y., Ma, Y. T., Zhang, J. Y., and Xie, X. (2020). COVID-19 and the cardiovascular system. *Nat. Rev. Cardiol.* 17, 259–260.

Zhou, P., Yang, X. L., Wang, X. G., Hu, B., Zhang, L., Zhang, W., et al. (2020). A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature* 579, 270–273. doi: 10.1038/s41586-020-2012-7

Zuo, T., Zhang, F., Lui, G. C. Y., Yeoh, Y. K., Li, A. Y. L., Zhan, H., et al. (2020). Alterations in gut microbiota of patients With COVID-19 during time of hospitalization. *Gastroenterol* S0016-S5085, 34701–34706. doi: 10.1053/j.gastro.2020.05.048

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Public Health

OPINION
published: 17 September 2020
doi: 10.3389/fpubh.2020.559135

Prevention and Control of COVID-19 in Italian Prisons: Stringent Measures and Unintended Consequences

Lara Tavoschi[1], Roberto Monarca[2,3], Ruggero Giuliani[4], Alessio Saponaro[5], Stefano Petrella[6], Roberto Ranieri[4], Filipa Alves da Costa[7], Carina Ferreira-Borges[7] and Linda Montanari[8]*

[1] Department of Translational Research and New Technologies in Medicine and Surgery, University of Pisa, Pisa, Italy, [2] Health Without Barriers, The European Federation for Prison Health, Viterbo, Italy, [3] Department of Infectious Diseases, Viterbo Local Health Unit, Viterbo, Italy, [4] Infectious Diseases Service, Penitentiary Health System, San Paolo University Hospital, Milan, Italy, [5] Emilia Romagna Region, Bologna, Italy, [6] Penitentiary Health System, Modena Local Health Unit, Modena, Italy, [7] WHO European Office for Prevention and Control of Noncommunicable Diseases (NCD Office), Moscow, Russia, [8] Public Health Unit, European Monitoring Centre for Drugs and Drug Addiction, Lisbon, Portugal

Keywords: COVID-19, pandemic, prison, Italy, prevention and control, people who use drugs (PWUD)

OPEN ACCESS

Edited by:
Longxiang Su, Peking Union Medical College Hospital (CAMS), China

Reviewed by:
Heino Johann Stöver, Frankfurt University of Applied Sciences, Germany

****Correspondence:***
Lara Tavoschi
lara.tavoschi@unipi.it

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Public Health

Received: *05 May 2020*
Accepted: *14 August 2020*
Published: *17 September 2020*

Citation:
Tavoschi L, Monarca R, Giuliani R, Saponaro A, Petrella S, Ranieri R, Alves da Costa F, Ferreira-Borges C and Montanari L (2020) Prevention and Control of COVID-19 in Italian Prisons: Stringent Measures and Unintended Consequences. Front. Public Health 8:559135. doi: 10.3389/fpubh.2020.559135

INTRODUCTION

The need to integrate prisons and other custodial settings in the comprehensive response to COVID-19 epidemic was recently advocated (1) and WHO as recently issued guidance to support members states in this direction (2).

The COVID-19 pandemic has been particularly dramatic in Italy, one of the first countries to be affected in Europe, with more than 200,000 cases reported as of 22/4/2020 (3). Within the country, the northern regions, including Lombardy and Emilia Romagna, were the epicenter of the epidemic and massive efforts were put in place to contain its spread. The custodial system has been part of this wider endeavor, as prison healthcare services are managed by Ministry of Health in Italy, although with differences across regions due to healthcare decentralization.

THE PRISON SETTING

Prisons are settings of higher risk for COVID-19 infections as confined conditions, especially in a context of overcrowding, are one of the biggest challenges for controlling the spread of the infection. Italy is the third country in Europe per prison density with an occupational rate of 120% for 61,230 prison population at 29/2/2020 (4). People in prison are more vulnerable to COVID-19 because of their underlying health conditions with disproportionally higher rates of acute and chronic physical and mental illnesses, including cardiovascular diseases, diabetes and chronic respiratory diseases, and frequently facing greater exposure to risks such as smoking, poor hygiene and weaker immune defense to stress (5).

RESPONSE MEASURES IN PRISON SETTINGS

Avoiding COVID-19 spread into the custodial system is the primary objective of an effective strategy tailored to prisons. In the early stage of the epidemic a rapid scale-up of prevention and control measures was implemented in the northern regions in close coordination with relevant health authorities. Triage and syndromic screening were set-up for all individuals

entering prison premises, including staff, visitors and incoming detainees. Dedicated areas for triaging were identified and in 77% (151/197[1]) of existing institutions temporary tensile-structures were put in place. Collection of biological samples and access to laboratory facilities was ensured as per standard community protocols. Areas for medical isolation (dedicated wings, single detention rooms, COVID-19 prison hub) of close contacts/suspects/confirmed cases were designated and provided with adequate protective measures, in order to minimize risks of transmission within prison and to allow for management of mild COVID-19 cases. Severe cases were transferred to referral tertiary hospitals in the community. Adequate supply of personal protective equipment and disinfectants was managed in collaboration with Civil Protection Agency. As the epidemic spreads across the country, national guidance was also issued (6).

ADDITIONAL MEASURES

The Ministry of Justice early on in the epidemic response issued organizational recommendations and stringent limitation on admission to prison premises, in particular restricting access to essential staff and banning visitors including relatives (7). The measure was deemed necessary to minimize COVID-19 introduction risks, and swiftly implemented. To mitigate its impact, alternatives to face-to-face visits were gradually implemented. Yet, when enacted, the measure sparked unrest across the whole country, with serious events occurring in some institutions. In Modena and Milan prisons people assaulted pharmacies ingesting large quantity of opioids used to treat drug addictions. Nine persons died in 1 day in the Modena's prison (8), although was ongoing at the time of writing. Like in many other countries, people with drug use disorders are overrepresented in prison, with 28% of the entire Italian prison population falling in this category and 34% being incarcerated for drug related crimes at 31/12/2018 (4, 9). Alternative measures to incarcerations (house arrest for up to 5,000 individuals) currently implemented within the COVID-19 response framework to reduce the number of inmates (10), might largely involve the sub-groups of drug users and people incarcerated for drug related crimes (9). Therefore, while COVID-19 prevention remains a primary concern, appropriate management of addiction, including linkage to community drug and social services, is necessary to respond to released individuals' health needs.

[1]As of 4th of April 2020.

CURRENT ASSESSMENT OF IMPACT

Still, the data currently available at this early stage suggest that the introduction of prevention and control measures had a positive impact on the spread of COVID-19 into and within the Italian prison system. More than 8-week into the epidemic with thousands of cases reported, only few cases occurred in prison. In Lombardy (11) and in Emilia-Romagna regions, where prison services swiftly implemented thorough prevention and control protocols, respectively 19 and 14 COVID-19 cases were reported, including one death, as of 22/4/2020.

CONCLUSIONS

While COVID-19 cases in the prison system are unavoidable, heightened attention along with stringent and comprehensive measures are needed when country-wide lockdown measures are relaxed. The COVID-19 pandemic calls on us to fulfill the principle "prison health is public health" in order to protect the well-being of people in prison and their community, uphold equity and avoid serious organizational, security and safety dangers resulting from outbreaks occurring in this setting.

AUTHOR CONTRIBUTIONS

LT and LM conceived the manuscript. All authors contributed to manuscript drafting, read, and approved the final version.

REFERENCES

1. Kinner SA, Young JT, Snow K, Southalan L, Lopez-Acuña D, Ferreira-Borges C, et al. Prisons and custodial settings are part of a comprehensive response to COVID-19. *Lancet Public Health*. (2020) 5:e188–9. doi: 10.1016/S2468-2667(20)30058-X
2. World Health Organization. *Preparedness, Prevention and Control of COVID-19 in Prisons And Other Places Of Detention. Interim Guidance, 15 March 2020*. Geneva: WHO (2020). Available online at: http://www.euro.who.int/_data/assets/pdf_file/0019/434026/Preparedness-prevention-and-control-of-COVID-19-in-prisons.pdf
3. Ministero della Salute. *Covid-19-Situazione in Italia*. (2020) Available online at: http://www.salute.gov.it/portale/nuovocoronavirus/dettaglioNotizieNuovoCoronavirus.jsp?lingua=italiano&menu=notizie&p=dalministero&id=4399 (accessed April 30, 2020).
4. Ministero della Giustizia. *Statistiche*. Available online at: https://www.giustizia.it/giustizia/it/mg_1_14.page
5. Azienda Regionale Sanità Toscana. *La Salute dei Detenuti in Italia: i Risultati di Uno Studio Multicentrico*. Florence: ARS Toscana (2015). Available online at: https://www.ars.toscana.it/files/pubblicazioni/Volumi/2015/carcere_2015_definitivo.pdf
6. Mancinelli R, D'Ancona FP, Libianchi S, Mustazzolu A, Tavoschi L, Pantosti A. *Indicazioni per la prevenzione e il controllo di COVID-19 negli istituti penitenziari e in altre strutture con limitazione delle libertá personali per adulti e minori*. Notiziario dell'Istituto Superiore di Sanitá (2020).
7. Ministero della Giustizia. *Decreti, Circolari, Direttive, Provvedimenti e Note*. Available online at: https://www.giustizia.it/giustizia/it/mg_1_8.page?all=true&viewcat=csdc_tipologia3&selectedNode=0_62
8. ANSA. *Rivolta in Carcere a Modena, 9 Detenuti Morti*. Protesta alla Dozza. (2020). Available online at: https://www.ansa.it/emiliaromagna/notizie/2020/03/09/rivolta-in-carcere-a-modena-inchiesta_feb67370-2f6f-459c-ad23-d0b496ec18e9.html
9. Antigone. *Il Carcere Secondo la Costituzione. XV Rapporto di Antigone sulle Condizioni di Detenzione. Spaccio, Traffico e Dipendenze. Il Motore Primo Della Carcerazione*. Antigone (2019). Available online at: http://www.antigone.it/quindicesimo-rapporto-sulle-condizioni-di-detenzione/il-lavoro-2/
10. Decreto Legge 17 Marzo 2020. n. 18. *Misure di Potenziamento del Servizio Sanitario Nazionale e di Sostegno Economico per Famiglie, Lavoratori E Imprese Connesse all'Emergenza Epidemiologica da COVID-19*. (2020)

Available online at: https://www.gazzettaufficiale.it/eli/gu/2020/03/17/70/sg/pdf

11. World Health Organization. *Experience of Health Professionals, Police Staff and Prisoners in Italy Informs WHO COVID-19 Guidelines for Prisons*. WHO (2020). Available online at: https://www.euro.who.int/en/countries/italy/news/news/2020/5/experience-of-health-professionals,-police-staff-and-prisoners-in-italy-informs-who-covid-19-guidelines-for-prisons

Disclaimer: CF-B is a staff member of the WHO, and FAC is a WHO consultant. The authors alone are responsible for the views expressed in this publication and these do not necessarily represent the decisions or the stated policy of the World Health Organization.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

PERSPECTIVE
published: 17 September 2020
doi: 10.3389/fmicb.2020.02112

Flexible, Functional, and Familiar: Characteristics of SARS-CoV-2 Spike Protein Evolution

Dianita S. Saputri[1†], Songling Li[1†], Floris J. van Eerden[2], John Rozewicki[1], Zichang Xu[1], Hendra S. Ismanto[1], Ana Davila[1], Shunsuke Teraguchi[1,2], Kazutaka Katoh[1] and Daron M. Standley[1,2]*

[1] Department of Genome Informatics, Research Institute for Microbial Diseases, Osaka University, Suita, Japan,
[2] Immunology Frontier Research Center, Osaka University, Suita, Japan

The SARS-CoV-2 S protein is a major point of interaction between the virus and the human immune system. As a consequence, the S protein is not a static target but undergoes rapid molecular evolution. In order to more fully understand the selection pressure during evolution, we examined residue positions in the S protein that vary greatly across closely related viruses but are conserved in the subset of viruses that infect humans. These "evolutionarily important" residues were not distributed evenly across the S protein but were concentrated in two domains: the N-terminal domain and the receptor-binding domain, both of which play a role in host cell binding in a number of related viruses. In addition to being localized in these two domains, evolutionary importance correlated with structural flexibility and inversely correlated with distance from known or predicted host receptor-binding residues. Finally, we observed a bias in the composition of the amino acids that make up such residues toward more human-like, rather than virus-like, sequence motifs.

Keywords: flexibility, host like, molecular evolution, phylogenetics, SARS-CoV-2, spike protein, structural modeling, structure alignment

OPEN ACCESS

Edited by:
Hirokazu Kimura,
Gunma Paz University, Japan

Reviewed by:
Koo Nagasawa,
Eastern Chiba Medical Center, Japan
Masahiro Ito,
Ritsumeikan University, Japan
Yoshiyuki Suzuki,
Nagoya City University, Japan

***Correspondence:**
Daron M. Standley
standley@biken.osaka-u.ac.jp

[†]These authors have contributed equally to this work

Specialty section:
This article was submitted to Virology, a section of the journal Frontiers in Microbiology

Received: *03 July 2020*
Accepted: *11 August 2020*
Published: *17 September 2020*

Citation:
Saputri DS, Li S, van Eerden FJ, Rozewicki J, Xu Z, Ismanto HS, Davila A, Teraguchi S, Katoh K and Standley DM (2020) Flexible, Functional, and Familiar: Characteristics of SARS-CoV-2 Spike Protein Evolution. Front. Microbiol. 11:2112. doi: 10.3389/fmicb.2020.02112

INTRODUCTION

Over 200 viruses are known to infect humans (Woolhouse et al., 2012). Among recent human virus outbreaks, three (SARS-CoV-1, MERS-CoV, and SARS-CoV-2) have arisen from beta coronaviruses. The close interaction between pathogen and host can be a driving force for molecular evolution. This is nowhere more apparent than on the surfaces of the viruses themselves. The characteristic crown-shaped spikes, for which coronaviruses are named, enable binding to and entering host cells, and also provide camouflage from the host immune system. The ectodomain – the most outer part of the spike (S) protein – consists of two functional subunits, the receptor-binding subunit (S1) and the membrane fusion subunit (S2) (**Figure 1A**). The S1 subunits are highly variable across genera, while the S2 subunits are much more conserved. These differences reflect their distinct functions: Whereas the S1 regions engage with receptors on the surfaces of host cells, the primary function of S2 is to mediate fusion with host cell membranes. The S1 subunit is located within the N-terminus of the S protein and can be further divided into an N-terminal domain (NTD) and a C-terminal domain, which, in itself, can be divided into a receptor-binding domain (RBD) located at the apex of the protein when viewed from the side and two additional domains

connecting it to the NTD (Wang et al., 2020). In SARS-CoV-2, the RBD contains a receptor-binding motif (437–508) that contains host receptor-binding residues. The structural domains of the S protein wind around each other such that the three RBDs and NTDs constitute a nearly continuous surface at the apex of the trimeric protein (**Figure 1B**).

The targets of the S1 NTD and RBD can differ greatly among beta coronaviruses. For example, the NTD can recognize sugar derivatives in human coronavirus (HCoV)-HKU1 and HCoV-OC43, which facilitate attachment to host cells; in mouse hepatitis coronavirus (MHV), the NTD binds to the host protein carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1). Meanwhile, the RBD binds hACE2 in SARS-CoV-1 and SARS-CoV-2, but binds aminopeptidase N (APN) and dipeptidyl peptidase 4 (DPP4) in HCoV-229E and MERS-CoV, respectively (Wang et al., 2020). This large variability in binding partners suggests that NTD and RBD are sites of intense evolutionary pressure.

In order to better understand this evolutionary pressure, we estimated the evolutionary importance of residue positions in SARS-CoV-2 by comparing the amino acid diversity of each position to that of equivalent positions in closely related viruses that infect non-human hosts. We found that evolutionary importance was high in the NTD and RBD. Moreover, within these domains, residues with high evolutionary importance could be characterized by three features: they are more *flexible* (when simulated by molecular dynamics) than surrounding residues, they occur in or around known *functionally important* host – protein binding sites, and their sequences are much more self-like or *familiar* to the host immune system than other residues.

Estimating Evolutionary Importance

It is possible to infer evolutionarily important residues in the S protein by observing sites that are conserved within a given branch of the phylogenetic tree but vary among different branches. To construct a phylogenetic tree, 20 SARS-CoV-2 S protein sequences, 6 close outgroups that infect bat and pangolin, and several sequences from other lineages of beta coronavirus (SARS-CoV, MERS-CoV, and HCoV-HKU1) were collected. Amino acid sequences were aligned by MAFFT (Katoh and Standley, 2013), and a neighbor-joining (Saitou and Nei, 1987) tree was estimated to roughly visualize the phylogenetic relationship (**Figure 1C**). We subsequently estimated the sequence diversity at each position using 9,827 SARS-CoV-2 sequences (https://www.gisaid.org; after filtering out those with many ambiguous bases and/or fragmentary sequences). These sequences were compared only with the close outgroups that infect bat and pangolin. The diversity for the combination of human + outgroup and for the human group alone was compared. We defined "evolutionary importance" as the difference:

$$\text{diversity(human + outgroup)} - \text{diversity(human)}$$

assuming that this difference reflects the change in evolutionary pressure when this virus is transmitted to humans. This resulted in three levels of importance: low (0), medium (1), and high (2) as indicated in the heatmap projected onto the molecular surface of the S protein. While low- and medium-importance positions were distributed widely across the S protein surface, most of the positions with high importance were confined to two domains: the NTD and the RBD (**Figure 1D**).

For comparison, local evolutionary rate in the human-infecting lineage was estimated by (100 AA) sliding window analysis. The evolutionary rate in this lineage is proportional to the evolutionary distance between the present-day sequences infecting humans and the common ancestor of human-infecting and bat-infecting lineages. The average distance, D, between these two points was estimated, for each window, by the relative rate test (Sarich, 1969):

$$D = \frac{1}{2}\{d(h,p) + d(h,b) - d(b,p)\}$$

where, h, p, b denote human, pangolin, and bat, respectively; pairwise distance was computed using the Poisson correction, $\mathrm{d}(,) = -\ln(1 - \mathrm{x/y})$, where x is the number of differences between sequences, and y is the number of sites. A high evolutionary rate in this lineage was clearly observed near hACE2-binding sites (**Figure 1E**). This observation is consistent with the site-specific diversity observed in the evolutionary importance; such sites have apparently changed radically upon transfer to humans and have been highly conserved thereafter. However, regarding the NTD region, the evolutionary rate was not estimated to be as high as the RBD in the human lineage. This local evolutionary rate analysis has three limitations: (1) it uses average rates of multiple adjacent residues, (2) it does not consider conservation within the human-infecting lineage, and (3) it cannot distinguish changes in a specific lineage from background changes in the same region. By defining evolutionary importance as we have above, we clearly observe sites that are specifically conserved in the lineage infecting humans.

Evolutionary Importance of Flexible Regions

It has been established that in SARS-CoV-1 and SARS-CoV-2, the RBD undergoes a large conformational change from the "closed" state to the "open" state upon engagement with hACE2 (Walls et al., 2020; Wrapp et al., 2020). In order to visualize flexible regions in the SARS-CoV-2 S protein, we carried out molecular dynamic simulations of the S protein in the open conformation followed by the root-mean square fluctuation (RMSF) analysis (**Figure 2A**). Not surprisingly, the most flexible parts of the protein were in loop regions. We observed that the beta sheet cores of both the S1 NTD and RBD domains were stable, as was most (but not all) of the S2 subunits. There were two exceptions with a higher RMSF: residue alanine 684 is part of the furin cleavage site (RRAR), which has been shown to be essential for infection of human lung cells (Hoffmann et al., 2020) and residues 830–840, which constitute a fusion peptide. Overall, we observed a nearly linear correlation between evolutionary importance and mean RMSF of these regions (Spearman correlation 0.30, $p < 2.2 \times 10^{-16}$) (**Figure 2B**). It is possible that flexibility in the NTD and RBD loops provide an induced-fit binding mechanism, wherein loop regions rearrange

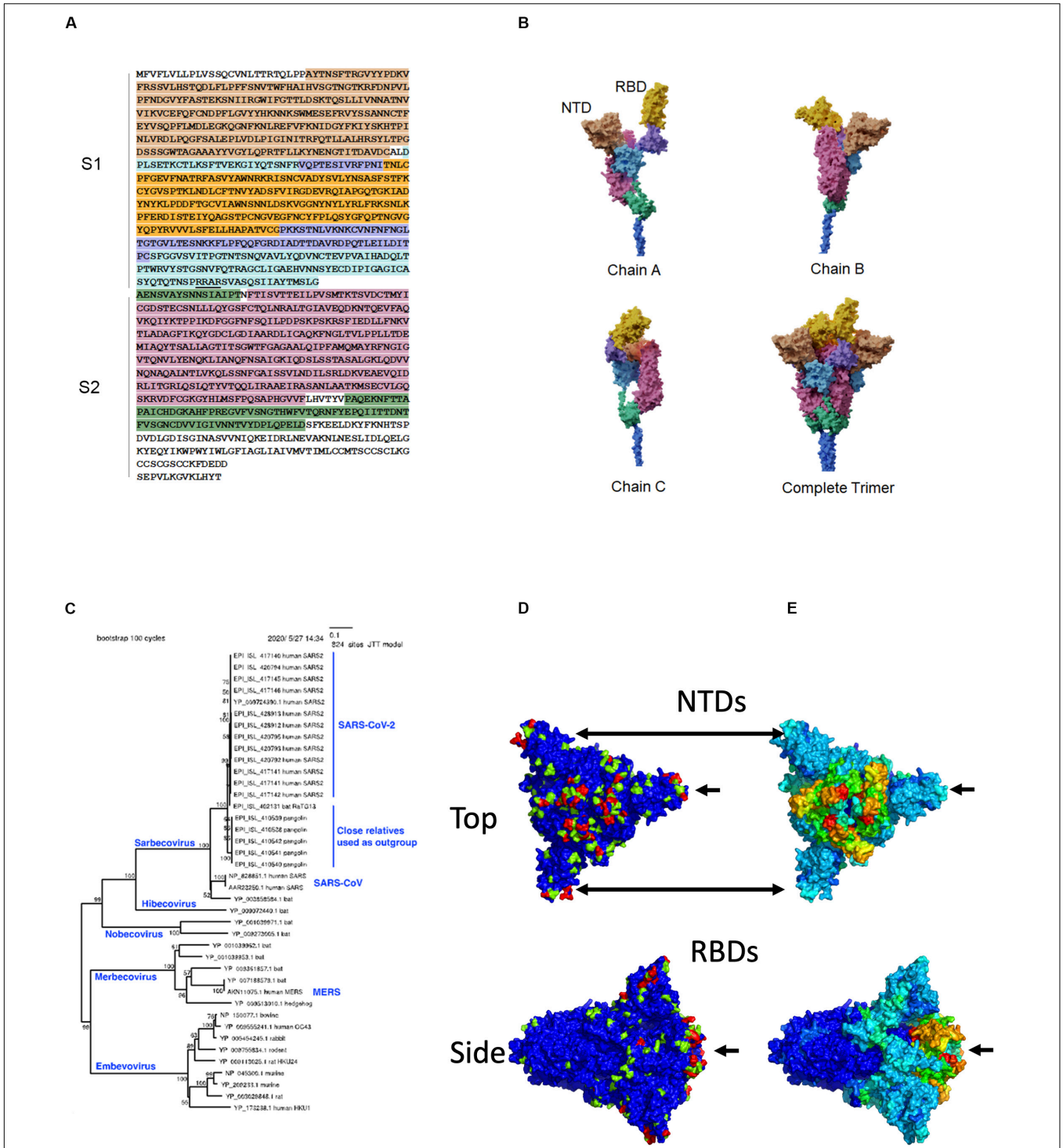


FIGURE 1 | Sequence, structure, and evolution of SARS-CoV-2 spike protein. **(A)** The sequence of the ectodomain is obtained from Uniprot with accession number P0DTC2 and is shaded according to six structural domains that correspond to those of **(B)**. The trimeric ectodomain can be divided into S1 and S2 subunits. In this figure, S1 is defined as residues 27–700 and S2 as 701–1,146. The S1 subunit can be further divided into the N-terminal domain (NTD, residues 27–291) and C-terminal domain (CTD, residues 294–700). The S1 CTD can further be divided into smaller structural domains, including the receptor-binding domain (RBD), in magenta, which is composed of residues 333–526 in this figure. The furin "RRAR" cleavage site at the end of S1 is underlined. **(B)** The three chains are interwoven to form the complete trimer. Here, the overall model was based on PDB entry 6vsb with NTD domains replaced by those of entry 5×4s. **(C)** Phylogenetic tree of closely related spike proteins from viruses that infect humans along with a representative outgroup. **(D)** Molecular surface of spike with evolutionary importance represented as a heatmap at three levels: low (blue), medium (green), and high (red). **(E)** Molecular surface of spike with evolutionary rate represented as a heatmap on a scale from lowest (blue) to highest (red).

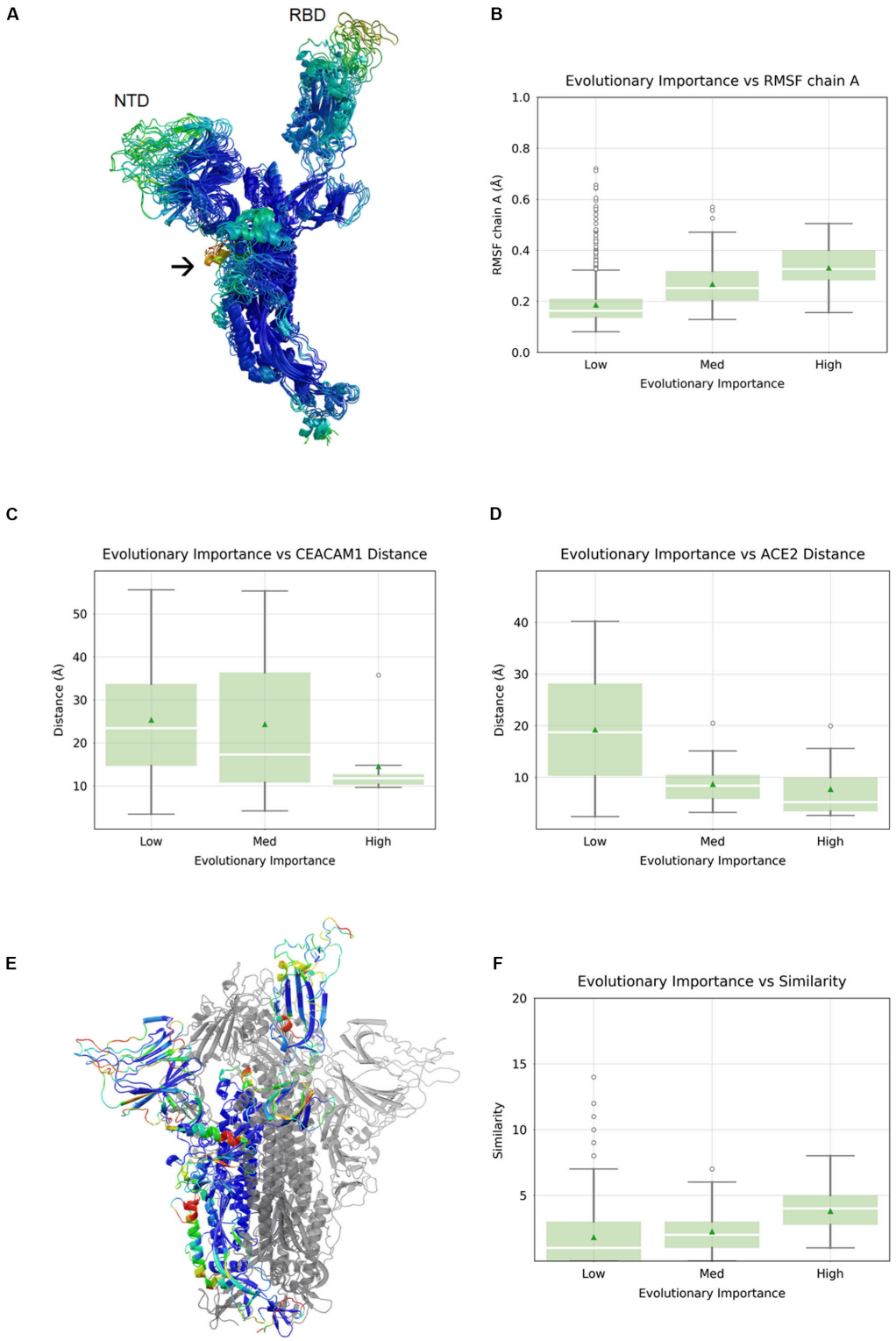


FIGURE 2 | Correlation between evolutionary importance and other properties. **(A)** The ectodomain of the spike protein colored according to the root-mean square fluctuation (RMSF), with the scale going from blue (low RMSF), via green and yellow to red (high RMSF). The NTD and RBD are labeled, and an arrow indicates the location of the fusion peptide. **(B)** Boxplot of RMSF as a function of evolutionary importance indicating that evolutionary importance is higher in flexible regions. **(C)** Boxplot of distance from carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) interface, as inferred from superposition on MHV, for residues in the three groups of evolutionary importance. **(D)** Boxplot of distance from ACE2-binding site for residues in the three groups of evolutionary importance. **(E)** Similarity is represented as a heatmap (from low to high: blue, cyan, green, yellow, orange, red) on a single chain of the spike protein with the other two chains shown in gray. **(F)** Boxplots showing similarity to human proteins highest for evolutionary important residues.

in order to properly bind to their host receptors. To explore this idea further, we analyze the relationship between evolutionary importance and distance from host receptor-binding sites below.

Evolutionary Importance and Proximity to Functional Binding Sites

The SARS-CoV-2 RBD mediates host cell entry by binding to hACE2. While the target of the SARS-CoV-2 NTD is still unknown, the high evolutionary importance in the NTD suggests a potential binding partner. Even without knowing the target of the NTD, we can assume that the location of the binding site is roughly conserved, and the distance of each residue in the NTD from this location using the NTDs of other viruses as proxies was measured. We can, of course, perform a similar and more precise analysis in the RBD using the known RBD–ACE2 complex crystal structure (Lan et al., 2020). When we compared the evolutionary importance in SARS-CoV-2 S1-NTD with the distance to the MHV NTD–CEACAM1 interface (Peng et al., 2011), we observed a negative correlation with distance (Spearman correlation -0.19, $p = 1.8 \times 10^{-3}$) (**Figure 2C**). The fact that evolutionary importance is higher in residues located near the equivalent site suggests that SARS-CoV-2 S1-NTD may have retained host binding and that the location of the binding site is roughly conserved. We compared the evolutionary importance with the distance to the hACE2-binding site (Yan et al., 2020) and observed that the evolutionary importance was higher in residues located near the ACE2 interface, consistent with its functional importance (Spearman correlation -0.38, $p < 8.8 \times 10^{-8}$) (**Figure 2D**). Taken together, we can say that evolutionary important residues occur often in flexible loops in or near known or putative virus – host binding interfaces.

Evolutionary Importance of Host-Like Sequences

Since the outer parts of the virus are most exposed to the host immune system, we aimed to look for their similarity with human cell surface proteins, as such similarity may indicate immune evasion. We carried out local alignment of all five-residue sequence fragments with a representative set of 507 human cell surface proteins as annotated by the Cell Surface Protein Atlas (Bausch-Fluck et al., 2015). The local sequence similarity was computed for each SARS-CoV-2 residue using rigorous matching criteria for each fragment. This analysis revealed several hotspots of similarity, including the NTD and RBD (**Figure 2E**). We quantified the relationship between similarity to human cell surface proteins and evolutionary importance and found that the similarity was highest for residues with the greatest importance (Spearman correlation 0.13, $p < 7.7 \times 10^{-6}$) (**Figure 2F**).

DISCUSSION

We estimated evolutionary importance based on generally diverse residue positions that are conserved within the SARS-CoV-2. We observed that such residues were primarily restricted to two domains, the NTD and RBD, both of which have host receptor-binding functions in a number of closely related viruses. Interestingly, these "important" residues were more flexible than less important residues, suggesting that the flexibility is a characteristic of rapid molecular evolution. Moreover, the residues tended to cluster near or within known or predicted host receptor-binding sites. This is not surprising, since the Evolutionary Trace method, on which our simple definition of evolutionary importance was based, has widely been used for predicting protein – protein interactions (Wodak and Mendez, 2004).

The fact that the NTD includes many evolutionary important residues strongly hints at a role in host receptor binding. Moreover, the correlation of evolutionary importance with distance from the known CEACAM1-binding site implies that the location of the binding site might be conserved. A recent report that anti-NTD antibodies can be neutralizing (Chi et al., 2020) supports this notion. We observed that evolutionary important residues appeared to be biased toward "human-like" sequence motifs more than other residues suggesting that they may have more potential to evade the immune system through mimicking the host protein. Although the sequence data on SARS-CoV-2 is still limited, the patterns may provide clues about the identity of targeted human cell surface receptors.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

DS carried out structural analysis of domains in related viruses. SL constructed a full-length model of SARS-CoV-2 S protein. FE performed molecular dynamics simulations of S protein. JR developed software for structural alignment. ZX carried out S protein docking. HI performed sequence analysis of SARS-CoV-2 S protein. AD carried out immunogenic (epitope) prediction on S protein. ST performed statistics calculations. KK did phylogenetic analysis. DS conceived of the project and wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This research was supported by a Grant-in-Aid for Scientific Research by the Japan Society for the Promotion of Science (JSPS) and by the Platform Project for Supporting Drug Discovery and Life Science Research [Basis for Supporting Innovative Drug Discovery and Life Science Research (BINDS)] from AMED under Grant No. 17am0101108j0001. Molecular Dynamics calculations were performed using machine time

on the TSUBAME supercomputer granted by the Tokyo Institute of Technology.

ACKNOWLEDGMENTS

We would like to thank Prof. Tatsuo Shioda, Osaka University, for his helpful suggestions and careful reading of the manuscript. We would also like to thank all members of the Systems Immunology Lab for helpful discussions regarding the preparation of the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.02112/full#supplementary-material

REFERENCES

Bausch-Fluck, D., Hofmann, A., Bock, T., Frei, A. P., Cerciello, F., Jacobs, A., et al. (2015). A mass spectrometric-derived cell surface protein atlas. *PLoS One* 10:e0121314. doi: 10.1371/journal.pone.0121314

Chi, X., Yan, R., Zhang, J., Zhang, G., Zhang, Y., Hao, M., et al. (2020). A potent neutralizing human antibody reveals the N-terminal domain of the Spike protein of SARS-CoV-2 as a site of vulnerability. *BioRxiv [Preprint]* doi: 10.1101/2020.05.08.083964

Hoffmann, M., Kleine-Weber, H., and Pohlmann, S. (2020). A Multibasic Cleavage Site in the Spike Protein of SARS-CoV-2 Is Essential for Infection of Human Lung Cells. *Mol. Cell.* 78, 779-784 e775. doi: 10.1016/j.molcel.2020.04.022

Katoh, K., and Standley, D. M. (2013). MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol. Biol. Evol.* 30, 772–780. doi: 10.1093/molbev/mst010

Lan, J., Ge, J., Yu, J., Shan, S., Zhou, H., Fan, S., et al. (2020). Structure of the SARS-CoV-2 spike receptor-binding domain bound to the ACE2 receptor. *Nature* 581, 215–220. doi: 10.1038/s41586-020-2180-5

Peng, G., Sun, D., Rajashankar, K. R., Qian, Z., Holmes, K. V., and Li, F. (2011). Crystal structure of mouse coronavirus receptor-binding domain complexed with its murine receptor. *Proc. Natl. Acad. Sci. U.S.A.* 108, 10696–10701. doi: 10.1073/pnas.1104306108

Saitou, N., and Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4, 406–425.

Sarich, V. M. (1969). Pinniped origins and the rate of evolution of carnivore albumins. *Systematic Zoology* 18, 286–295. doi: 10.2307/2412325

Walls, A. C., Park, Y. J., Tortorici, M. A., Wall, A., Mcguire, A. T., and Veesler, D. (2020). Structure, Function, and Antigenicity of the SARS-CoV-2 Spike Glycoprotein. *Cell* 181, 281-292 e286. doi: 10.1016/j.cell.2020.02.058

Wang, Q., Zhang, Y., Wu, L., Niu, S., Song, C., Zhang, Z., et al. (2020). Structural and Functional Basis of SARS-CoV-2 Entry by Using Human ACE2. *Cell* 181, 894-904 e899. doi: 10.1016/j.cell.2020.03.045

Wodak, S. J., and Mendez, R. (2004). Prediction of protein-protein interactions: the CAPRI experiment, its evaluation and implications. *Curr. Opin. Struct. Biol.* 14, 242–249. doi: 10.1016/j.sbi.2004.02.003

Woolhouse, M., Scott, F., Hudson, Z., Howey, R., and Chase-Topping, M. (2012). Human viruses: discovery and emergence. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 367, 2864–2871. doi: 10.1098/rstb.2011.0354

Wrapp, D., Wang, N., Corbett, K. S., Goldsmith, J. A., Hsieh, C. L., Abiona, O., et al. (2020). Cryo-EM structure of the 2019-nCoV spike in the prefusion conformation. *Science* 367, 1260–1263. doi: 10.1126/science.abb2507

Yan, R., Zhang, Y., Li, Y., Xia, L., Guo, Y., and Zhou, Q. (2020). Structural basis for the recognition of SARS-CoV-2 by full-length human ACE2. *Science* 367, 1444–1448. doi: 10.1126/science.abb2762

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Medicine

BRIEF RESEARCH REPORT
published: 18 September 2020
doi: 10.3389/fmed.2020.549860

Direct Clinical Evidence Recommending the Use of Proteinase K or Dithiothreitol to Pretreat Sputum for Detection of SARS-CoV-2

Jing Peng[1†], Yanjun Lu[1†], Juan Song[2†], Bruce A. Vallance[3], Kevan Jacobson[3], Hong Bing Yu[3] and Ziyong Sun[1*]*

[1] Department of Laboratory Medicine, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China, [2] Department of Gastroenterology & Endocrinology, Wuhan No. 9 Hospital, Wuhan, China, [3] Department of Pediatrics, BC Children's Hospital Research Institute, University of British Columbia, Vancouver, BC, Canada

OPEN ACCESS

Edited by:
Zisis Kozlakidis,
International Agency for Research on Cancer (IARC), France

Reviewed by:
Fei Yu,
Agricultural University of Hebei, China
Ahmed Mostafa,
National Research Centre, Egypt

***Correspondence:**
Hong Bing Yu
hby@mail.ubc.ca
Ziyong Sun
zysun@tjh.tjmu.edu.cn

[†] These authors have contributed equally to this work

Specialty section:
This article was submitted to Infectious Diseases – Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: 07 April 2020
Accepted: 27 August 2020
Published: 18 September 2020

Citation:
Peng J, Lu Y, Song J, Vallance BA, Jacobson K, Yu HB and Sun Z (2020) Direct Clinical Evidence Recommending the Use of Proteinase K or Dithiothreitol to Pretreat Sputum for Detection of SARS-CoV-2. Front. Med. 7:549860. doi: 10.3389/fmed.2020.549860

One of the primary tools for diagnosing COVID-19 is the nucleic acid-based real-time reverse transcriptase-polymerase chain reaction (RT-PCR) test performed on respiratory specimens. The detection rate of SARS-CoV-2 in lower respiratory specimens (such as sputum) is higher than that for upper respiratory specimens (such as nasal and pharyngeal swabs). However, sputum specimens are usually quite viscous, requiring a homogenization process prior to nucleic acid (NA) extraction for RT-PCR. Sputum specimens from COVID-19 and non-COVID-19 patients were treated with four commonly used reagents—saline, N-acetyl-L-cysteine (NALC), proteinase K (PK), and dithiothreitol (DTT), prior to NA extraction. These reagents were then compared for their performance in diagnosing COVID-19 in real clinical practice. The detection rate of SARS-CoV-2 in PK- or DTT-treated sputum was comparable, and higher than that in sputum treated with NALC or saline. While there was a 4.8% (1/21) false negative rate for the PK- and DTT-treated sputum, neither treatment showed any false positive cases among patients with non-COVID diseases. Moreover, sputum pretreated with saline, NALC, PK or DTT showed higher detection rates of SARS-CoV-2 as compared to pharyngeal swabs. Taken together, we provide direct evidence recommending the use of PK or DTT to pretreat sputum samples to facilitate SARS-CoV-2 detection by clinical laboratories. Moreover, our methods should help to standardize the procedure of processing sputum specimens and improve the ability to detect SARS-CoV-2 in these samples.

Keywords: sputum, detection of SARS-CoV-2, COVID-19, proteinase K (PK), DTT

Coronavirus disease 2019 (COVID-19) outbreak caused by SARS-Cov-2 was declared a global pandemic on Mar 11, 2020. One of the primary tools for diagnosing COVID-19 is the nucleic acid-based, real-time reverse transcriptase-polymerase chain reaction (RT-PCR) test, performed on respiratory specimens. The detection rate of SARS-CoV-2 in lower respiratory specimens (such as sputum) is higher than that obtained with upper respiratory specimens (such as nasal and pharyngeal swabs) (1). However, sputum samples often contain a large amount of mucus and

are viscous, resulting in the trap of virus containing cell components within the mucus. This will prevent nuclei acid (NA) extraction reagents from accessing to these components, leading to low yield of RNA. Without being homogenized sufficiently, sputum samples can have multiple adverse effects, such as introducing cross-contamination to the automatic nucleic acid extraction instrument, and causing pipetting errors, clot formation, or failed amplification (2). Surprisingly, a number of kits used for detecting SARS-CoV-2 in sputum lack specific instructions on how to homogenize sputum samples before NA extraction.

Several reagents have been used to homogenize sputum samples, such as proteinase K (PK), dithiothreitol (DTT), and N-acetyl-L-cysteine (NALC). PK is a stable serine alkaline protease, and has broad substrate specificity (2, 3). It is often used to digest abundant proteins present in sputum samples, and preferentially degrades ester and peptide bonds next to the C-termini of hydrophobic, sulfuric, or aromatic amino acids. This digestion process inactivates nucleases that could degrade DNA or RNA during isolation and purification procedures. DTT has a very low redox potential, and is able to quantitatively reduce disulfide bonds and maintain monothiol in a reduced state. As a source of the reactive sulfhydryl groups, NALC is mucolytic (4). It prevents the formation of intramolecular and intermolecular disulfide bonds in sputum samples. By disrupting disulfide bonds, both DTT and NALC are widely used to liquefy mucus (5, 6). The proper homogenization and liquefaction of mucus by PK, DTT, and NALC will help to remove substances that inhibit amplification, as well as increase the yield of extracted RNA. This will ultimately improve the detection of virus RNA by RT-PCR. Using spiked sputum samples, a previous study showed that PK-DNase method was ideal for homogenizing sputum samples prior to RT-PCR for the detection of Middle East respiratory syndrome coronavirus (MERS-CoV) (7). Sputum appeared to be a good clinical specimen in patients at the early stage of SARS infection (8), and it also outperformed nasal/pharyngeal swabs in detecting other respiratory viruses, such as respiratory syncytial virus, parainfluenza virus, and human metapneumovirus (9). More recently, SARS-CoV-2 is shown to be more readily detected in sputum samples than in throat swabs of convalescent COVID-19 patients (10). Despite these findings, clinical assessment is lacking regarding how sputum should be pretreated for its best performance in diagnosing SARS-CoV-2 infections. To address this, we treated clinical sputum specimens with four commonly used reagents—saline, NALC, PK, and DTT, prior to NA extraction, and compared their performance in diagnosing COVID-19 in real practice.

METHODS

Sputum Collection

A total of 68 sputum specimens were collected from adult patients admitted to Tongji Hospital, Huazhong University of Science and Technology (Wuhan, China) between Jan 28 and Mar 2, 2020. Of these patients, 21 were diagnosed as SARS-CoV-2 positive (SARS-CoV-2^+) based on clinical symptoms (fever, cough, and dyspnea), computed tomography (CT) and/or RT-PCR results from pharyngeal swabs. The remaining 47 patients had diseases unrelated to COVID-19. This study was approved by the ethics committee of Tongji Hospital (TJ-C2030).

Sputum Treatment

All sputum specimens were repeatedly pipetted up and down every 5 min, and vortexed for 30 min. Each sputum specimen was aliquoted into four Eppendorf tubes (500 μl per tube), followed by the addition of 500 μl of saline, NALC (0.5 g/100 ml, Sinopharm Chemical Reagent Co. Ltd, freshly made), PK (1 g/l, TianLong Science and Technology Co., Ltd., Xi'an, China), and DTT (Sputasol, Oxoid Microbiological Products) into each tube. Samples were kept upright at room temperature for 30 min, and pipetted up and down once at the 15 min interval, until completely liquefied.

RNA Extraction and RT-PCR Test

Liquefied sputum samples were centrifuged, and the supernatants (250 μl) were used for RNA extraction on a fully automated nucleic acid extraction system 9600E, using the NA extraction kit from Tianlong Science & Technology (TianLong Science and Technology Co., Ltd., Xi'an, China). The RT-PCR was performed with a SARS-CoV-2 nucleic acid detection kit according to the manufacturer's instructions (Da'an Gene Co., Ltd. of Sun Yat-Sen University, Guangzhou, China, approved by National Medical Products Administration). This kit detects *ORF1ab* and N genes from SARS-CoV-2. Samples with cycle threshold values (Ct-values) for both genes $\leq$ 40, or a Ct-value of only one gene $\leq$ 40, repeated twice, were defined as SARS-CoV-2^+. Samples with Ct-values for both genes $>$ 40, or not showing an amplification curve for either gene, were defined as SARS-CoV-2 negative.

RESULTS

With the sputum samples collected from the 47 patients having non-COVID-19 diseases, no amplification curves were observed for either the *ORF1ab* or N gene under any treatment conditions (saline, NALC, PK, and DTT), suggesting no SARS-CoV-2 in these samples. In contrast, RT-PCR tests of all the sputum samples from the SARS-CoV-2^+ patients showed Ct-values for both genes $<$ 40, or Ct-values of one gene $<$ 40 (repeated twice), under at least one treatment condition. Ct-values of the N gene from saline-, NALC-, PK-, and DTT-pretreated sputum samples were 38.3 $\pm$ 9.8, 34.2 $\pm$ 8.0, 34.0 $\pm$ 7.4, 33.4 $\pm$ 6.8, respectively, with the NALC-, PK-, and DTT-pretreated groups significantly lower than the saline-treated group (**Figure 1A**). Ct-values of the *ORF1ab* gene from these treatments were 41.4 $\pm$ 8.7, 39.3 $\pm$ 9.2, 39.3 $\pm$ 8.8, 36.9 $\pm$ 8.0, respectively, with the DTT-pretreated group significantly different from the saline-treated group (**Figure 1B**).

According to the positive criteria described in the methods section, pretreatment of sputum samples with NALC, PK, and DTT increased the detection of SARS-CoV-2^+ cases to 85.7% (18/21), 95.2% (20/21), and 95.2% (20/21), respectively, as compared to the 52.4% (11/21) obtained with saline pretreated sputum (see **Table 1**). Of the 8 mucopurulent sputum samples,

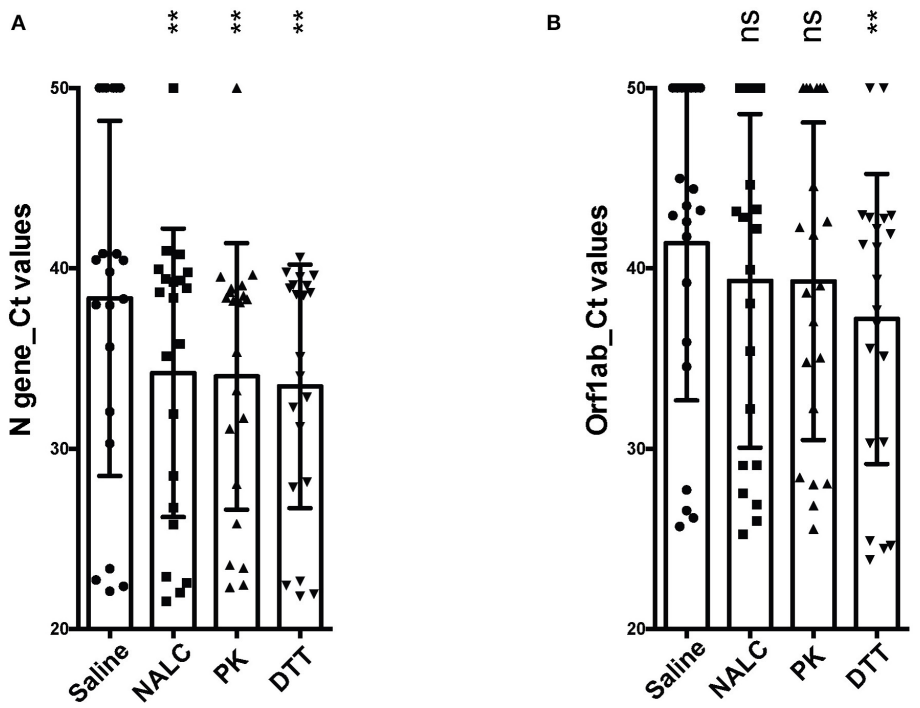


FIGURE 1 | Comparison of different pretreatments for detection of SARS-CoV-2 from sputum. **(A)** Ct-values of the N gene from clinical sputum specimens under different treatments. **(B)** Ct-values of the *ORF1ab* gene from clinical sputum specimens under different treatments. Statistical differences were calculated using one-way ANOVA with *post-hoc* turkey multiple comparison test. For clinical sputum specimens showing no amplication curves, Ct-values of 50 were arbitrarily assigned to calculate statistical differences. $^{**}p < 0.01$; ns, not significant. NALC, N-Acetyl-L-Cysteine; PK, Proteinase K; DTT, Dithiothreitol.

only 1 was found to be SARS-CoV-2 negative when treated with PK or DTT. Notably, the amount of sputa required for the detection of SARS-CoV-2 by NALC, PK, or DTT treatments was as low as 0.2 ml.

Of the 21 SARS-CoV-2$^+$ patients, 15 also had pharyngeal swabs collected within 1 day of sputum collection (i.e., when sputum was used for RT-PCR test), with only 40% (6/15) of these patients identified as SARS-CoV-2$^+$ based on testing the swabs by RT-PCR.

DISCUSSION

Based on previous experience with other respiratory viruses, the USA CDC has recommended the use of DTT to pretreat sputum for the detection of SARS-CoV-2. However, there is a lack of direct clinical evidence supporting this suggestion or the use of other chemicals to pretreat sputum prior to performing RT-PCR tests for SARS-CoV-2.

We examined how different pretreatments affected SARS-CoV-2 detection from sputum specimens. Our RT-PCR assay showed a higher sensitivity for detecting the N gene, similar to that reported by others (11, 12). This likely results from the relative amplification differences between the *ORF1ab* or N genes, as the N gene has a much higher level of subgenomic mRNA (13). The homogenization of sputum by PK and DTT appeared to be very thorough, as these pretreatments increased the positive rates by ∼32.8% as compared to saline-treated sputum. Similar to our report, the detection of human avian influenza A (H7N9) virus from sputum samples is also improved by PK and DTT pretreatment (14). Pretreating sputum samples with NALC also improved the detection of SARS-CoV-2$^+$ cases, with freshly made NALC improving the detection rate by 15% as compared to 1-day-old NALC (data not shown). However, the detection rate of SARS-CoV-2 in NALC-treated sputum was lower than that obtained with PK- or DTT-treated sputum. One plausible reason for this could be that PK and DTT are able to digest mucous protein more completely than NALC, resulting in increased concentration of extracted RNA. Supporting this, DTT (a dithiol having two redox-active cysteine residues) is more effective than NALC (a monothiol) in reducing sputum elasticity (15, 16).

Sputum pretreated with saline, NALC, PK, or DTT showed a higher detection rate than when assessing pharyngeal swabs, a finding similar to that outlined in two recent reports (1, 17). While there was only a 4.8% (1/21) false negative rate for the PK- or DTT-treated sputum samples, neither treatment caused any false positive cases among patients with non-COVID-19 diseases. These data suggest that testing sputum may be a preferred approach to diagnosing COVID-19, as well as differentiating SARS-CoV-2 from other prevalent viral infections that cause similar symptoms (18). Such approaches could potentially

TABLE 1 | Summary of sputum test results and sputum characteristics.

Patient ID	Treatment					Sputum characteristics		
	Saline	NALC	PK	DTT	Swabs	Viscosity	Appearance	Amount (ml)
1	−	+	+	+	NA	Moderate	Mucopurulent	2.5
2	−	−	+	+	NA	High	Mucopurulent	1.6
3	−	+	+	+	−	Moderate	Mucoid	2
4	−	+	+	+	−	Mild	Mucoid	1.5
5	−	+	+	+	NA	High	Mucoid	8
6	+	+	+	+	+	Moderate	Mucopurulent	6
7	+	+	+	+	+	Mild	Blood-tinged	1.2
8	+	+	+	+	+	Moderate	Blood-tinged	1.4
9	+	−	+	+	−	Mild	Mucoid	0.8
10	−	+	+	+	−	High	Mucopurulent	0.4
11	−	+	+	+	−	Moderate	Mucoid	1.5
12	−	+	+	+	NA	Mild	Mucoid	3
13	+	+	+	+	NA	Mild	Mucoid	4
14	+	+	+	+	−	High	Mucopurulent	0.5
15	+	+	+	+	NA	Mild	Mucoid	16
16	+	+	+	−	−	Moderate	Mucopurulent	1
17	−	+	+	+	+	High	Mucopurulent	0.2
18	+	+	+	+	−	High	Mucopurulent	2.5
19	+	+	+	+	+	Moderate	Blood-tinged	1.5
20	−	−	−	+	−	Moderate	Mucopurulent	1.2
21	+	+	+	+	+	High	Blood-tinged	2

A total of 21 sputum specimens were obtained from SARS-CoV-2 positive patients. Saline, NALC, PK, and DTT treatments of sputum were able to detect 52.4% (11/21), 85.7% (18/21), 95.2% (20/21), and 95.2% (20/21) of SARS-CoV-2$^+$ cases, respectively. Pharyngeal swabs were able to detect 40.0% (6/15) of SARS-CoV-2 positive cases. NA, not available.

decrease the pressure on critical care resources in hospitals where multiple pharyngeal swabs are often required to rule in/out COVID-19. Currently, many point-of-care tests (POCT) have already been developed for SARS-CoV-2 detection. These POCT tests will not only significantly reduce time of testing, but also help to optimize clinical management and increase patient satisfaction (19). While these tests are most likely effective with nasal/pharyngeal swabs or aspirates, it may not work well with non-homogenized sputum. Our findings thus provide a very promising scenario whereby sputum specimens pretreated with PK or DTT, in conjunction with these POCT tests, could significantly increase the accuracy of diagnosing COVID-19 as well as provide rapid diagnosis.

This study was limited to the single collection of sputum from a small number of SARS-CoV-2$^+$ inpatients with moderate to severe infections. Further studies should include more patients, particularly those with mild infections, as well as collect sputum at least twice from each patient. Also, in some cases, there is a limited availability of sputum from COVID-19 patients. In a study with a larger population, ∼33% of COVID-19 patients produced sputum (20), and the majority of COVID-19 patients may produce no or very limited amount of sputum. However, since the amount of sputum required for this assay is as low as 0.2 ml, we anticipate that pretreating sputum samples with PK or DTT will facilitate SARS-CoV-2 detection from patients who fail to produce abundant sputum.

CONCLUSION

In summary, while it is known that sputum samples usually outperform nasal/pharyngeal swabs in detecting respiratory viruses, including SARS-CoV-2, the performance of sputum samples in the diagnosis of COVID-19 can be further affected by the way how sputum was pretreated. We provide direct clinical evidence recommending the use of PK or DTT to pretreat sputum to facilitate SARS-CoV-2 detection in clinical laboratories. This recommendation could be further confirmed with more diverse clinical samples, ultimately improving the procedure of processing sputum specimens for their best performance in detecting SARS-CoV-2.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

This study was approved by the ethics committee of Tongji Hospital (TJ-C2030). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

JP, HY, and ZS had full access to all of the data in the study and take responsibility for the integrity of the data. JP, YL, JS, HY, and ZS: concept and design, acquisition, analysis, or interpretation of data. JP and HY: drafting of the manuscript. BV and KJ: critical revision of the manuscript for important intellectual content. HY: statistical analysis. ZS: obtained funding. HY and ZS: supervision. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by grants from National Mega Project on Major Infectious Disease Prevention (Grant Nos. 2017ZX10103005-007-001, 2017ZX10103005-007-002). The sponsors had no role in the design and conduct of the study, collection, management, analysis, and interpretation of the data, preparation, review, or approval of the manuscript, and decision to submit the manuscript for publication.

REFERENCES

1. Wang W, Xu Y, Gao R, Lu R, Han K, Wu G, et al. Detection of SARS-CoV-2 in different types of clinical specimens. *JAMA.* (2020) 323:1843–44. doi: 10.1001/jama.2020.3786
2. Wilson D, Yen-Lieberman B, Reischl U, Warshawsky I, Procop GW. Comparison of five methods for extraction of *Legionella pneumophila* from respiratory specimens. *J Clin Microbiol.* (2004) 42:5913–6. doi: 10.1128/JCM.42.12.5913-5916.2004
3. Sweeney PJ, Walker JM. Proteinase K (EC 3.4.21.14). *Methods Mol Biol.* (1993) 16:305–11. doi: 10.1385/0-89603-234-5:305
4. Samuni Y, Goldstein S, Dean OM, Berk M. The chemistry and biological activities of N-acetylcysteine. *Biochim Biophys Acta.* (2013) 1830:4117–29. doi: 10.1016/j.bbagen.2013.04.016
5. Cleland WW. Dithiothreitol, a new protective reagent for Sh groups. *Biochemistry.* (1964) 3:480–2. doi: 10.1021/bi00892a002
6. Saraswathy Veena V, Sara George P, Jayasree K, Sujathan K. Comparative analysis of cell morphology in sputum samples homogenized with dithiothreitol, N-acetyl-L cysteine, Cytorich®red preservative and in cellblock preparations to enhance the sensitivity of sputum cytology for the diagnosis of lung cancer. *Diagn Cytopathol.* (2015) 43:551–8. doi: 10.1002/dc.23266
7. Sung H, Yong D, Ki CS, Kim JS, Seong MW, Lee H, et al. Comparative evaluation of three homogenization methods for isolating Middle East respiratory syndrome coronavirus nucleic acids from sputum samples for real-time reverse transcription PCR. *Ann Lab Med.* (2016) 36:457–62. doi: 10.3343/alm.2016.36.5.457
8. Chan KH, Poon LL, Cheng VC, Guan Y, Hung IF, Kong J, et al. Detection of SARS coronavirus in patients with suspected SARS. *Emerg Infect Dis.* (2004) 10:294–9. doi: 10.3201/eid1002.030610
9. Falsey AR, Formica MA, Walsh EE. Yield of sputum for viral detection by reverse transcriptase PCR in adults hospitalized with respiratory illness. *J Clin Microbiol.* (2012) 50:21–4. doi: 10.1128/JCM.05841-11
10. Han H, Luo Q, Mo F, Long L, Zheng W. SARS-CoV-2 RNA more readily detected in induced sputum than in throat swabs of convalescent COVID-19 patients. *Lancet Infect Dis.* (2020) 20:655–6. doi: 10.1016/S1473-3099(20)30174-2
11. Chu DKW, Pan Y, Cheng SMS, Hui KPY, Krishnan P, Liu Y, et al. Molecular diagnosis of a novel coronavirus (2019-nCoV) causing an outbreak of pneumonia. *Clin Chem.* (2020) 66:549–55. doi: 10.1093/clinchem/hvaa029
12. Lu X, Wang L, Sakthivel SK, Whitaker B, Murray J, Kamili S, et al. US CDC real-time reverse transcription PCR panel for detection of severe acute respiratory syndrome coronavirus 2. *Emerg Infect Dis.* (2020) 26:1654–65. doi: 10.3201/eid2608.201246
13. Moreno JL, Zuniga S, Enjuanes L, Sola I. Identification of a coronavirus transcription enhancer. *J Virol.* (2008) 82:3882–93. doi: 10.1128/JVI.02622-07
14. Yu F, Qiu T, Zeng Y, Wang Y, Zheng S, Chen X, et al. Comparative evaluation of three preprocessing methods for extraction and detection of influenza A virus nucleic acids from sputum. *Front Med.* (2018) 5:56. doi: 10.3389/fmed.2018.00056
15. Rancourt RC, Tai S, King M, Heltshe SL, Penvari C, Accurso FJ, et al. Thioredoxin liquefies and decreases the viscoelasticity of cystic fibrosis sputum. *Am J Physiol Lung Cell Mol Physiol.* (2004) 286:L931–8. doi: 10.1152/ajplung.00352.2003
16. Nielsen H, Hvidt S, Sheils CA, Janmey PA. Elastic contributions dominate the viscoelastic properties of sputum from cystic fibrosis patients. *Biophys Chem.* (2004) 112:193–200. doi: 10.1016/j.bpc.2004.07.019
17. Lin C, Xiang J, Yan M, Li H, Huang S, Shen C. Comparison of throat swabs and sputum specimens for viral nucleic acid detection in 52 cases of novel coronavirus (SARS-Cov-2)-infected pneumonia (COVID-19). *Clin Chem Lab Med.* (2020) 58:1089–94. doi: 10.1515/cclm-2020-0187
18. Cascella M, Rajnik M, Cuomo A, Dulebohn SC, Di Napoli R. *Features, Evaluation and Treatment Coronavirus (COVID-19).* Treasure Island, FL: StatPearls (2020).
19. Sheridan C. Fast, portable tests come online to curb coronavirus pandemic. *Nat Biotechnol.* (2020) 38:515–8. doi: 10.1038/d41587-020-00010-2
20. Guan WJ, Ni ZY, Hu Y, Liang WH, Ou CQ, He JX, et al. Clinical characteristics of coronavirus disease 2019 in China. *N Engl J Med.* (2020) 382:1708–20. doi: 10.1101/2020.02.06.20020974

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Public Health

ORIGINAL RESEARCH
published: 18 September 2020
doi: 10.3389/fpubh.2020.567672

A Pattern Categorization of CT Findings to Predict Outcome of COVID-19 Pneumonia

*Chao Jin[1], Cong Tian[1], Yan Wang[1], Carol C. Wu[2], Huifang Zhao[1], Ting Liang[1], Zhe Liu[1], Zhijie Jian[1], Runqing Li[1], Zekun Wang[3], Fen Li[3], Jie Zhou[4], Shubo Cai[4], Yang Liu[5], Hao Li[6], Zhongyi Li[7], Yukun Liang[8], Heping Zhou[8], Xibin Wang[9], Zhuanqin Ren[10] and Jian Yang[1]**

[1] Department of Radiology, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, China, [2] Department of Thoracic Imaging, University of Texas M.D. Anderson Cancer Center, Houston, TX, United States, [3] Department of Radiology, The Eighth Hospital of Xi'an, Xi'an, China, [4] Department of Radiology, Xi'an Chest Hospital, Xi'an, China, [5] Department of Cardiology, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, China, [6] Department of Critical Care Medicine, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, China, [7] Department of Critical Care Medicine, Wuhan No.9 Hospital, Wuhan, China, [8] Department of Radiology, Ankang Center Hospital, Ankang, China, [9] Department of Radiology, Hanzhong Center Hospital, Hanzhong, China, [10] Department of Radiology, Baoji Center Hospital, Baoji, China

OPEN ACCESS

Edited by:
Zhongheng Zhang,
Sir Run Run Shaw Hospital, China

Reviewed by:
Lin Chen,
Jinhua Central Hospital, China
Mohamed Chakroun,
Hôpital Universitaire Fattouma Bourguiba, Tunisia
Yimin Li,
Guangzhou Medical University, China
Jordi Rello,
Vall D'Hebron Research Institute (VHIR), Spain

***Correspondence:**
Jian Yang
yj1118@mail.xjtu.edu.cn

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Public Health

Received: *30 May 2020*
Accepted: *27 August 2020*
Published: *18 September 2020*

Citation:
Jin C, Tian C, Wang Y, Wu CC, Zhao H, Liang T, Liu Z, Jian Z, Li R, Wang Z, Li F, Zhou J, Cai S, Liu Y, Li H, Li Z, Liang Y, Zhou H, Wang X, Ren Z and Yang J (2020) A Pattern Categorization of CT Findings to Predict Outcome of COVID-19 Pneumonia. Front. Public Health 8:567672. doi: 10.3389/fpubh.2020.567672

Background: As global healthcare system is overwhelmed by novel coronavirus disease (COVID-19), early identification of risks of adverse outcomes becomes the key to optimize management and improve survival. This study aimed to provide a CT-based pattern categorization to predict outcome of COVID-19 pneumonia.

Methods: One hundred and sixty-five patients with COVID-19 (91 men, 4–89 years) underwent chest CT were retrospectively enrolled. CT findings were categorized as Pattern 0 (negative), Pattern 1 (bronchopneumonia pattern), Pattern 2 (organizing pneumonia pattern), Pattern 3 (progressive organizing pneumonia pattern), and Pattern 4 (diffuse alveolar damage pattern). Clinical findings were compared across different categories. Time-dependent progression of CT patterns and correlations with clinical outcomes, i.e.„ discharge or adverse outcome (admission to ICU, requiring mechanical ventilation, or death), with pulmonary sequelae (complete absorption or residuals) on CT after discharge were analyzed.

Results: Of 94 patients with outcome, 81 (86.2%) were discharged, 3 (3.2%) were admitted to ICU, 4 (4.3%) required mechanical ventilation, 6 (6.4%) died. 31 (38.3%) had complete absorption at median day 37 after symptom onset. Significant differences between pattern-categories were found in age, disease severity, comorbidity and laboratory results (all $P < 0.05$). Remarkable evolution was observed in Pattern 0–2 and Pattern 3–4 within 3 and 2 weeks after symptom-onset, respectively; most of patterns remained thereafter. After controlling for age, CT pattern significantly correlated with adverse outcomes [Pattern 4 vs. Pattern 0–3 [reference]; hazard-ratio [95% CI], 18.90 [1.91–186.60], $P = 0.012$]. CT pattern [Pattern 3–4 vs. Pattern 0–2 [reference]; 0.26 [0.08–0.88], $P = 0.030$] and C-reactive protein [>10 vs. ≤10 mg/L [reference]; 0.31 [0.13–0.72], $P = 0.006$] were risk factors associated with pulmonary residuals.

Conclusion: CT pattern categorization allied with clinical characteristics within 2 weeks after symptom onset would facilitate early prognostic stratification in COVID-19 pneumonia.

Keywords: novel coronavirus disease, computed tomography, CT pattern, clinical outcome, pulmonary sequelae

INTRODUCTION

Since the latter part of December of 2019, an outbreak of respiratory disease caused by severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2) has become a pandemic (1). As of May 29, 2020, 5,704,736 laboratory-confirmed cases and 357,736 deaths have been reported (2). Numerous studies have revealed the epidemiological, clinical, and radiological characteristics of the novel coronavirus disease (COVID-19) (3–6). Despite the fact that more than 80% of infected patients manifest with only mild clinical symptoms (3), early identifying the risks of an adverse outcome remains the key to optimize management and improve survival. Previous studies found that advanced age and presence of comorbidity (e.g., cardiovascular disease or hypertension) were risk factors associated with an adverse outcome such as admission to intensive care unit (ICU), need for mechanical ventilation, or death (7, 8). In addition, some laboratory indicators e.g., elevated hypersensitive troponin I, leukocytosis, neutrophilia, lymphopenia, and elevated D-dimer were found to be linked with unfavorable clinical outcomes (7–9). Presence of consolidation on computed tomography (CT) was also considered to be predictive of poor outcome in COVID-19 (10). Despite the above, the identification of early prognostic signs of COVID-19 remains of urgent importance due to the diversity in clinical and imaging findings as well as the severity and rapid progression of disease.

It is recognized that CT plays a central role in diagnosis and management of COVID-19 pneumonia (11–13). Reported CT findings of COVID-19 pneumonia included the ground glass opacities (GGO), consolidation, septal thickening mainly along the subpleural lungs or bronchovascular bundles or diffusely in the entire lungs (14). These are highly suggestive of lung organization response to injury from COVID-19 pneumonia, similar to radiological findings in the diffuse alveolar damage (DAD) and organizing pneumonia (OP) (15). Pathological studies also observed DAD in patients who succumbed to COVID-19 (16). Previous studies have demonstrated a decreased survival rate of 35–50% in DAD, while most patients with OP had better prognosis (15). In this regard, a pattern categorization of COVID-19 pneumonia, i.e., DAD and OP patterns may help the prognostic stratification. Based on the prior study regarding influenza A (H1N1) pneumonia (17), Lee also suggested a pattern categorization of COVID-19, i.e., bronchopneumonia, OP and DAD (18). A rapid progression of OP-like injury in Severe Acute Respiratory Syndrome (SARS) was considered to be predictive of a protracted clinical course (19). This may suggest a progressive subtype of OP pattern. Based on the aforementioned knowledge, a CT pattern categorization of COVID-19 pneumonia, i.e., bronchopneumonia, OP, progressive OP and DAD may have potential prognostic implications, e.g., adverse outcome, clinical course with recovery. As healthcare systems in many countries are overwhelmed with COVID-19 patients, improved prediction of the course of the disease based on early findings can assist with improved utilization of limited resources. To this end, this study aimed to investigate the prognostic significance of a CT pattern categorization in conjunction with the clinical indicators on clinical outcome and pulmonary sequelae in COVID-19.

METHODS

Participants

The internal review board approved this retrospective study. Written informed consent was waived with approval. Between January 22, and March 16, 2020, 172 laboratory-confirmed COVID-19 patients who underwent chest CT were collected from eight hospitals in China. The cases were from four regions (Xi'an, $n = 80$; Baoji, $n = 10$; Ankang, $n = 18$; Hanzhong, $n = 17$) in Shaanxi province and Wuhan ($n = 47$) in Hubei province.

A case of COVID-19 was confirmed by a positive result on next-generation sequencing or real-time RT-PCR. The disease type, i.e., uncomplicated illness, mild pneumonia, severe pneumonia, critical illness (acute respiratory distress syndrome, sepsis or septic shock) was evaluated based on the criteria published by World Health Organization (WHO) (20).

All the patients were treated based on Diagnosis and Treatment Protocol for Novel Coronavirus Pneumonia (Trial Version 7) issued by National Health Commission of the People's Republic of China, which includes initiation of antivirals, interferon, Chinese herbal medications, supplemental oxygen as needed and hospitalization. The criteria for patient discharge with recovery included: (1) afebrile for >3 days, (2) improved respiratory symptoms, (3) chest imaging shows obvious resolution of inflammation, and (4) two consecutively negative nucleic acid test results (sampling interval ≥1 day) (21). The recommendations for discharged patients included (1) 14 days of isolation management and health monitoring; (2) follow-up hospital visits with a next-generation sequencing or real-time RT-PCR test and chest CT scan to detect whether there exist a positive return and/or pulmonary residuals excluding the underlying lesions on CT with linear opacities, and/or a few consolidation with/without GGO at 2 and 4 weeks after discharge (21).

CT Image Acquisition

All chest CT were acquired by using 16- or 64-multidector CT scanners (GE LightSpeed 16, GE VCT LightSpeed 64, GE Optima 680, GE Healthcare; Philips Brilliant 16, Philips Healthcare; Somatom Sensation 64, Somatom AS, Somatom Spirit, Siemens

TABLE 1 | Definition of COVID-19 pneumonic pattern based on CT findings.

CT pattern	Definition	CT findings
Pattern 0	Negative	None
Pattern 1	Bronchopneumonia pattern	• Discrete lesion with a peribronchial distribution • CT signs with GGO or consolidation, or tree-in-bud sign or nodular opacity (**Figure 3**) • Lung lobar involvement assessed by total CT score ≤5
Pattern 2	Organizing pneumonia pattern	• Multifocal lesions with a peripheral distribution predominantly in the middle to lower lung zones • CT signs with GGO or consolidation, and/or interlobular septal thickening (**Figure 4**) • Lung lobar involvement assessed by total CT score ≤6
Pattern 3	Progressive organizing pneumonia pattern	• Multiple lesions with a peripheral distribution predominantly in the middle to lower lung zones • CT signs with consolidation or GGO or mixed GGO and consolidation, and/or interlobular septal thickening (**Figure 5**) • Lung lobar involvement assessed by total CT score more than 6 and < 10
Pattern 4	Diffuse alveolar damage pattern	• Lesions with extensive distribution diffusely in the entire lungs • CT signs with consolidation mixed with or without GGO, and/or air bronchograms (**Figure 6**) • Lung lobar involvement assessed by total CT score more than or equal to 10

The primary CT signs (GGO, consolidation, linear opacity, interlobular septal thickening and air bronchograms) were included to define the CT patterns; while other signs e.g.„ pleural effusion, lymphadenopathy and so on were not considered due to the infrequency in each pattern. Negative refers to the no abnormality on CT.
GGO, ground glass opacities.

Healthcare). Patients were scanned in the supine position from the level of the upper thoracic inlet to the inferior level of the costophrenic angle with the following parameters: tube voltage of 120 kVp, current intelligent control (auto mA) of 30–300 mA, and slice thickness reconstructions of 0.625–1.5 mm.

Data Collection and Evaluation

We extracted the demographic data, clinical symptoms, and laboratory tests on admission from electronic medical records. The date of disease onset was defined as patients' reported date of symptom onset. The time intervals from symptom onset to each CT were determined. The primary clinical outcome was discharge or adverse outcome (admission to ICU, use of mechanical ventilation, or death). The secondary outcome was pulmonary sequelae, i.e., complete absorption or residuals on CT at the first follow-up visit after discharge.

All CT images and pattern categorization were independently evaluated by two experienced radiologists, respectively, with 4 and 10 years of pulmonary imaging experience, who were blinded to the clinical and laboratory data of patients. Prior to the evaluation, they were trained by a lecture- and literature-based session that explained CT findings (10–13), a chest imaging score assessing the degree of lobar involvement (22), and pattern categorizations (15, 17) of COVID-19. During the session, 209 CT images from 56 cases randomly selected from this study cohort were individually evaluated and then differences were discussed with a final consensus. The remaining CT images were first individually evaluated and then evaluated together 3 weeks after individual evaluation. Any difference was discussed with a final consensus. Individual evaluations were used for calculation of inter-observer agreement (see more in the **Supplementary Material**), and consensus evaluations were used for subsequent analysis.

CT findings including the presence and distribution of GGO, consolidation, linear opacity, pleural effusion and lymphadenopathy were evaluated. The degree of lobar involvement and total lung severity score were also evaluated (22). Based on the degree or area of involvement, each of the five lung lobes was scored of 0 for 0% lobe involvement, 1 for 1–25% lobe involvement, 2 for 26–50% lobe involvement, 3 for 51–75% lobe involvement, or 4 for 76–100% lobe involvement. A total severity score was calculated by summing the scores of the five lobes (range, 0–20).

CT pattern categorization was performed based on the above CT findings and total lung severity (15, 17) (**Table 1**). Receiver operating characteristic curve analysis was used to estimate the cutoff CT scores in discriminations of Pattern 2 vs. 3 and Pattern 3 vs. 4, respectively (see more in **Supplement Material**). In cases with two or more patterns, predominant pattern was designated.

Statistical Analysis

Continuous variables were represented as means and standard deviations, while categorical variables were expressed as counts and percentages. Differences of demographic, clinical and CT imaging characteristics across pattern groups were analyzed by dependent sample *t*-test, Chi-square test or Fisher's exact test as appropriate. Bonferroni correction was used in multiple comparisons. Chi-square test for trend was used to explore the time-dependent change of each CT pattern. Univariate Cox proportional-hazards regression was first used to explore the risk factors related to clinical adverse outcomes and pulmonary residuals. Multivariate Cox proportional-hazards regression with Kaplan-Meier curve plots were further used to explore the risk factors based on the significant variables in the above univariate analysis.

All statistical analyses were performed using SPSS 17.0 (SPSS; Chicago, IL, USA) and Medcalc 19.1.7 (MedCals Software Ltd.; Ostend, Belgium). $P < 0.05$ was considered statistically significant.

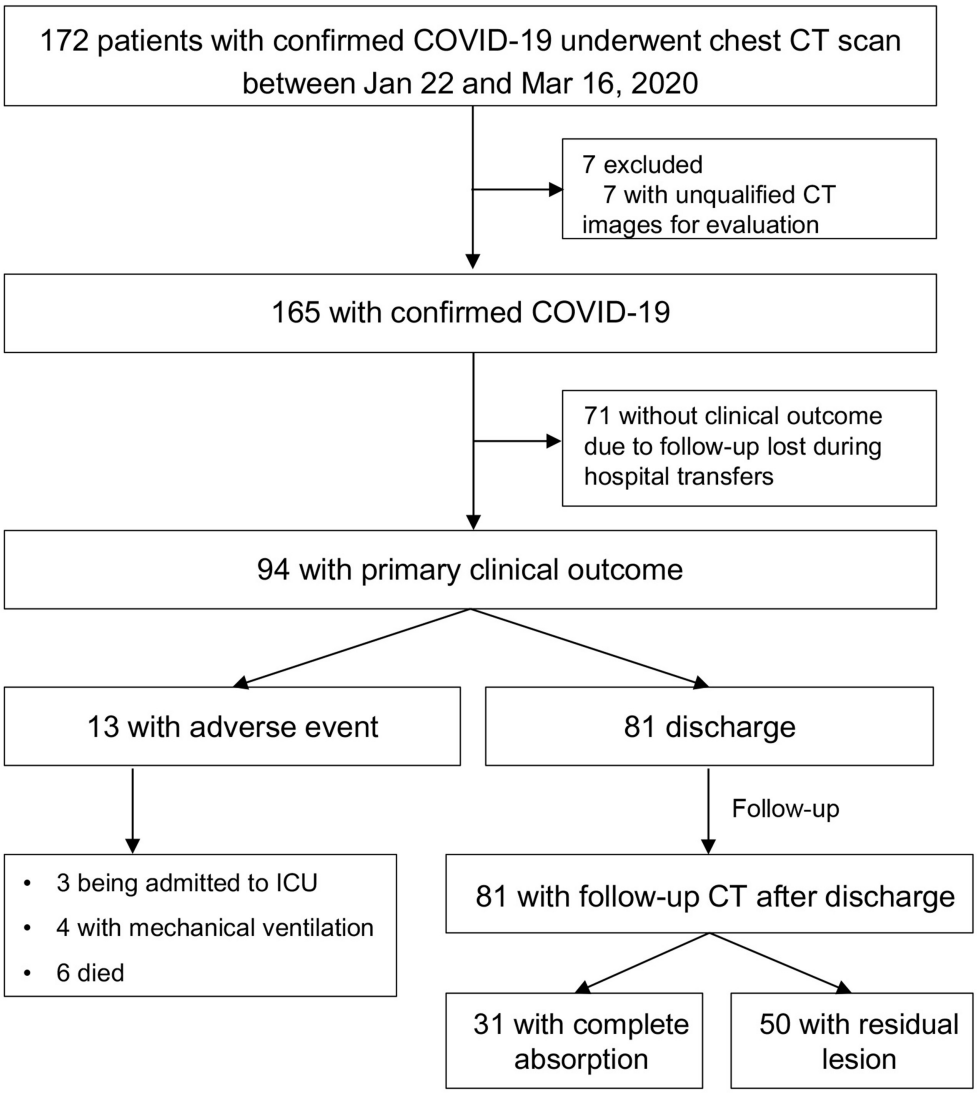


FIGURE 1 | Study flow diagram. COVID-19, coronavirus disease 2019; ICU, intensive care unit.

RESULT

Patient Demographic and Clinical Characteristics

Of 172 patients, 165 patients were included. As of 16 Mar 2020, 94 patients had clinical outcomes and 71 were follow-up lost without clinical outcome records due to hospital transfer (**Figure 1**). Of 94 patients, 81(86.2%) were discharged, 3(3.2%) were admitted to ICU, 4(4.3%) required mechanical ventilation, 6(6.4%) died. 31(38.3%) patients had complete absorption of lesions on CT after discharge. The median time from symptom onset to discharge was 21 (range, 10–41) days, and median times from symptom onset to being admitted to ICU, to requiring mechanical ventilation, and to death were 7 (range, 2–8) days, 8 (range, 8–49) days, and 33.5 (range, 7–39) days, respectively. The median times from symptom onset and from discharge to post-discharge CT scan were 37 (range, 14–58) days, 15 (range, 9–29) days, respectively.

Patients were categorized into five CT patterns based on the baseline CT: 7(4.3%) were Pattern 0, 36 (21.8%) were Pattern 1, 67 (40.6%) were Pattern 2, 32 (19.4%) were Pattern 3, and 23(13.9%) were Pattern 4. All the patients had 478 chest CT, 34 (21.2%) had 1 CT, 41 (23.6 %) had 2 CT, 39 (23.7%) had 3 CT, and 51 (31.5%) had more than 3 CT. The median time from symptom onset to baseline CT was 7 (range, 1–44) days.

Table 2 detailed the clinical characteristics and laboratory results of patients by CT pattern group. In the full cohort, the mean age was 49.5 (SD, 15.9; range, 4–89) years and there was no gender difference [91 [55.2%] men, 74 [44.8%] women].

TABLE 2 | Characteristics of COVID-19 pneumonia patients with various CT patterns.

Characteristic	All (n = 165)	Pattern 0 (n = 7)	Pattern 1 (n = 36)	Pattern 2 (n = 67)	Pattern 3 (n = 32)	Pattern 4 (n = 23)	P-value	Pattern 0 vs. Pattern 1	Pattern 1 vs. Pattern 2	Pattern 2 vs. Pattern 3	Pattern 3 vs. Pattern 4
								P-value	P-value	P-value	P-value
Age (years) [a]	49.5 ± 15.9	39.7 ± 13.7	47.4 ± 16.5	43.9 ± 14.7	56.7 ± 11.1	61.7 ± 14.7	**< 0.001**	0.253	0.266	**< 0.001**[†]	0.158
Male sex	91 (55.2)	3 (42.9)	26 (72.2)	28 (41.8)	17 (53.1)	17 (73.9)	**0.012**	0.129	**0.003**[†]	0.289	0.118
Disease severity							**< 0.001**	0.294	0.949	**< 0.001**[†]	0.014
Mild	111 (67.3)	7 (100)	31 (86.1)	58 (86.6)	13 (40.6)	2 (8.7)					
Severe	44 (26.7)	0	5 (13.9)	9 (13.4)	16 (50.0)	14 (60.9)					
Critical illness	10 (6.0)	0	0	0	3 (9.4)	7 (30.4)					
Comorbidity [b]	101 (61.2)	2 (28.6)	8 (22.2)	21 (31.3)	18 (56.2)	15 (65.2)	**0.002**	0.716	0.326	0.018	0.503
Clinical symptom on admission											
Fever	140 (84.8)	4 (57.1)	26 (72.2)	60 (89.6)	28 (87.5)	22 (95.7)	**0.020**	0.655	0.024	0.743	0.387
Fatigue	30 (18.2)	3 (42.9)	2 (5.6)	11 (16.4)	5 (15.6)	9 (39.1)	**0.008**	0.024	0.133	0.920	0.048
Pharyngalgia	18 (10.9)	2 (28.6)	4 (11.1)	9 (13.4)	2 (6.3)	1 (4.3)	0.347	0.248	>0.999	0.495	>0.999
Headache	6 (3.6)	0	2 (5.6)	4 (6.0)	0	0	0.602	>0.999	>0.999	0.301	–
Cough	96 (58.2)	4 (57.1)	16 (44.4)	42 (62.7)	17 (53.1)	17 (73.9)	0.195	0.687	0.075	0.365	0.118
Expectoration	36 (21.8)	1 (14.3)	6 (16.7)	18 (26.9)	3 (9.4)	8 (34.8)	0.129	>0.999	0.243	0.046	0.038
Chest congestion/breath shortness	34 (20.6)	0	2 (5.6)	9 (13.4)	13 (40.6)	10 (43.5)	**< 0.001**	>0.999	0.321	**0.002**[†]	0.832
Muscle soreness	8 (4.8)	0	2 (5.6)	4 (6.0)	1 (3.1)	1 (4.3)	>0.999	>0.999	>0.999	>0.999	>0.999
Nausea and vomiting	1 (0.6)	0	1 (2.8)	0	0	0	>0.999	–	>0.999	>0.999	–
Diarrhea	4 (2.4)	0	0	2 (3.0)	1 (3.1)	1 (4.3)	0.735	–	0.541	>0.999	>0.999
No symptom	5 (3.0)	0	3 (8.3)	2 (3.0)	0	0	0.365	>0.999	0.340	>0.999	–
Laboratory test on admission [c]											
Lymphocyte percentage (%)							**< 0.001**	0.280	0.097	**0.004**[†]	0.836
< 20	62 (38.0)	0	6 (16.7)	21 (31.8)	20 (62.5)	15 (65.2)					
≥20	101 (62.0)	6 (100)	30 (83.3)	45 (68.2)	12 (37.5)	8 (34.8)					
Monocyte percentage (%)							0.315	0.414	0.085	0.102	0.261
>10	39 (24.5)	1 (16.7)	12 (33.3)	12 (18.2)	10 (33.3)	4 (19.0)					
≤10	120 (75.5)	5 (83.3)	24 (66.7)	54 (81.8)	20 (66.7)	17 (81.0)					
Leukocyte count (10^9/L)							0.062	0.167	0.570	0.924	0.014
< 3.5	40 (24.5)	0	9 (25.0)	20 (30.3)	10 (31.2)	1 (4.3)					
≥3.5	123 (75.5)	6 (100)	27 (75.0)	46 (69.7)	22 (68.8)	22 (95.7)					
Alanine Aminotransferase (U/L)							0.065	0.554	0.102	0.200	0.945
>50	28 (17.4)	0	2 (5.6)	11 (16.9)	9 (28.1)	6 (27.3)					
≤50	133 (82.6)	6 (100)	34 (94.4)	54 (83.1)	23 (71.9)	16 (72.7)					
Aspartate Aminotransferase (U/L)							0.122	0.328	0.035	0.702	0.583
>40	32 (19.9)	1 (16.7)	2 (5.6)	14 (21.5)	8 (25.0)	7 (31.8)					
≤40	129 (80.1)	5 (83.3)	34 (94.4)	51 (78.5)	24 (75.0)	15 (68.2)					

(Continued)

TABLE 2 | Continued

Characteristic	All ($n = 165$)	Pattern 0 ($n = 7$)	Pattern 1 ($n = 36$)	Pattern 2 ($n = 67$)	Pattern 3 ($n = 32$)	Pattern 4 ($n = 23$)	*P*-value	Pattern 0 vs. Pattern 1	Pattern 1 vs. Pattern 2	Pattern 2 vs. Pattern 3	Pattern 3 vs. Pattern 4
								P-value	*P*-value	*P*-value	*P*-value
Creatine kinase (U/L)							**0.014**	0.014	0.022	0.429	0.038
>310	18 (11.8)	1 (16.7)	0	9 (13.6)	2 (7.7)	6 (31.5)					
≤310	134 (88.2)	5 (83.3)	35 (100)	57 (86.4)	24 (92.3)	13 (68.4)					
Neutrophil percentage (%)							**< 0.001**	0.391	0.080	0.232	0.043
>75	48 (29.4)	0	4 (11.1)	17 (25.8)	12 (37.5)	15 (65.2)					
≤75	115 (70.6)	6 (100)	32 (88.9)	49 (74.2)	20 (62.5)	8 (34.8)					
C-reactive protein (mg/L)							**0.002**	0.130	0.245	0.356	0.055
>10	96 (63.6)	1 (16.7)	17 (50.0)	38 (62.3)	23 (71.9)	17 (94.4)					
≤10	55 (36.4)	5 (83.3)	17 (50.0)	23 (37.7)	9 (28.1)	1 (5.6)					
Hemoglobin (g/L)							0.494	0.873	0.976	0.684	0.251
< 130	35 (22.4)	1 (16.7)	7 (19.4)	13 (19.7)	7 (23.3)	7 (38.9)					
≥130	121 (77.6)	5 (83.3)	29 (80.6)	53 (80.3)	23 (76.7)	11 (61.1)					
CT findings on admission											
CT signs											
GGO only	28 (17.0)	0	13 (36.1)	12 (17.9)	2 (6.3)	1 (4.3)	**0.005**	–	0.040	0.215	>0.999
Consolidation	17 (10.3)	0	5 (13.9)	6 (9.0)	3 (9.4)	3 (13.0)	0.880	–	0.510	>0.999	0.686
GGO and consolidation	51 (30.9)	0	10 (27.8)	16 (23.9)	10 (31.3)	15 (65.2)	**0.002**	–	0.664	0.436	**0.013**
Linear opacity	0	0	0	0	0	0	–	–	–	–	–
GGO and linear opacity	7 (4.2)	0	2 (5.6)	3 (4.5)	2 (6.3)	0	0.839	–	>0.999	0.657	0.504
Consolidation and linear opacity	5 (3.0)	0	1 (2.8)	4 (6.0)	0	0	0.618	–	0.665	0.301	–
Three mixed signs	50 (30.3)	0	5 (13.9)	26 (38.8)	15 (46.9)	4 (17.4)	**0.003**	–	**0.009**†	0.446	0.023
Lobe involvement							**< 0.001**	–	0.121	**0.008**†	0.632
Number of lobe affected < 3	52 (31.5)	7 (100)	18 (50.0)	23 (34.3)	3 (9.4)	1 (4.3)					
Number of lobe affected≥3	113 (68.5)	0	18 (50.0)	44 (65.7)	29 (90.6)	22 (95.7)					
CT severity score [a]	6.0 ± 4.4	0	3.3 ± 2.1	4.7 ± 2.7	7.5 ± 2.8	14.0 ± 2.9	**< 0.001**	**< 0.001**†	**0.005**†	**< 0.001**†	**< 0.001**†

Unless otherwise indicated, data are reported as the number of patients, with percentages in parentheses. a, data are reported as the mean ± standard derivation. b, 70% of patients had history of hypertension and diabetes mellitus while only 2 had pulmonary tuberculosis and 2 had chronic bronchitis. c, more than 91·5% of patients had all laboratory tests and a few were lack of one or two indicators. †, Significance at P < 0.0125 with Bonferroni correction. Abbreviations: Pattern 0 = negative; Pattern 1 = organizing pneumonia pattern; Pattern 2 = progressive organizing pneumonia pattern; Pattern 4 = diffuse alveolar damage pattern; GGO, ground glass opacity; Three mixed signs = GGO, consolidation and linear opacity. The bold value refers to P < 0.05.

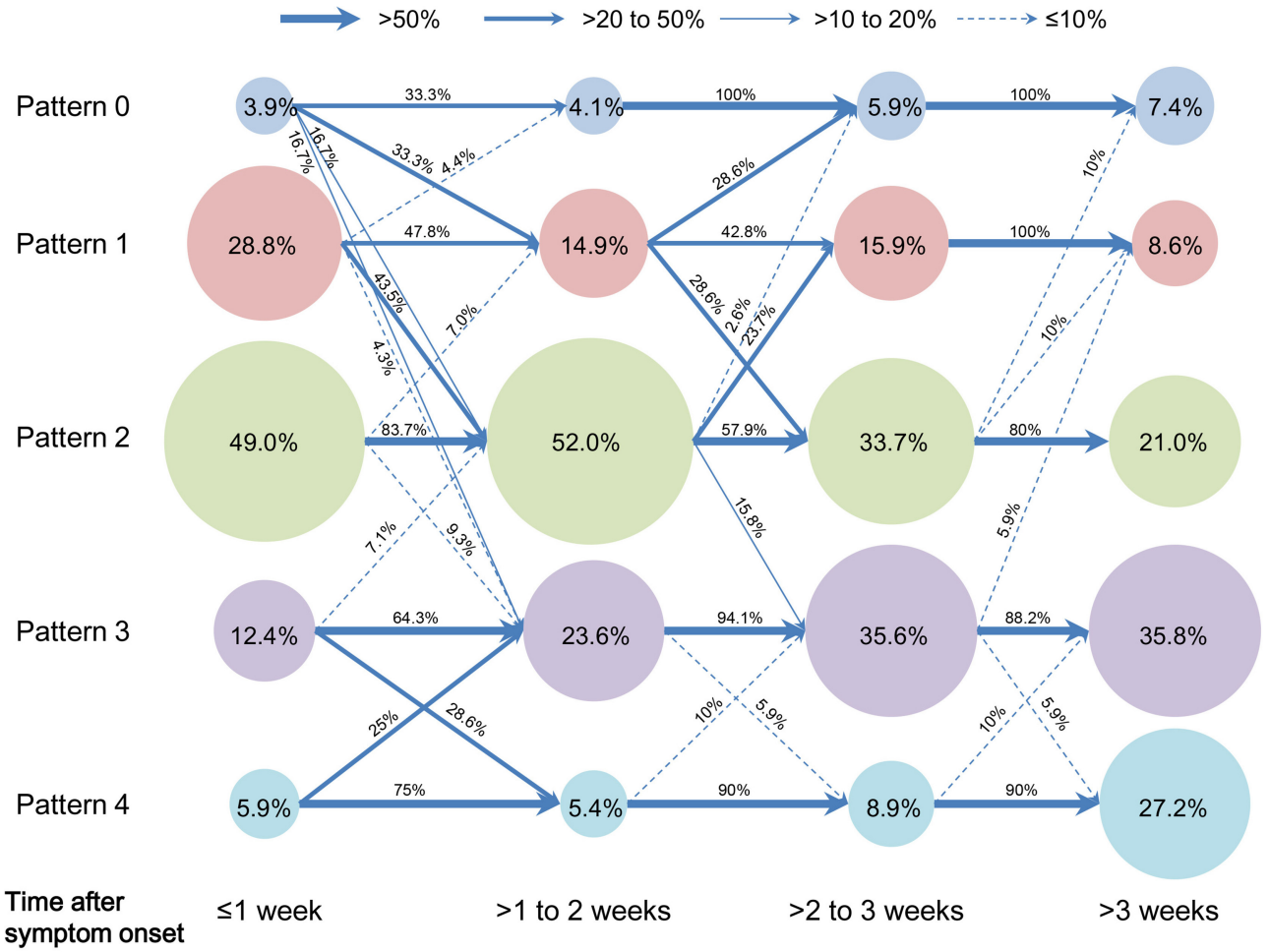


FIGURE 2 | Evolution of proportions of COVID-19 pneumonic CT pattern with the disease progression. Pattern data were designated to four time groups according to the time from symptom onset to CT scan: ≤1 week (CT pattern number = 153), >1–2 weeks (CT pattern number = 147), >2–3 weeks (CT pattern number = 101) and >3 weeks (CT pattern number = 77). The circular area indicated the proportion of CT pattern in each time group, e.g.„ proportions of Pattern 0 to 4 were 3.9, 28.8, 49.0, 12.4, and 5.9% during 1 week after symptom onset, respectively. Arrow line indicated the evolution of each CT pattern from a time group to the following, e.g.„ 33.3% of Pattern 1 progressed to Pattern 2 from 1 to 2 weeks after symptom onset; here four arrow line style denoted the categorization of evolution proportion, i.e.„ >50% (thick solid line), >20–50% (medium solid line), >10–20% (thin solid line), and ≤10% (dashed line).

Significant differences between pattern groups were found in age, sex distribution, disease severity, comorbidity, CT findings and laboratory results (all $P < 0.05$). Significant differences were also observed in multiple comparisons between any two patterns in one or more than one terms of age, sex distribution, disease severity, comorbidity, CT findings and laboratory results (all $P < 0.017$).

Evolution of COVID-19 Pneumonic CT Pattern With Disease Progression

Chi-square tests for trend indicated that as disease progresses from 1 to >3 weeks, proportions of Pattern 1 and 2 remarkably decreased, while those of Pattern 3 and 4 increased (all $P < 0.01$). With regard to evolution of CT pattern, Pattern 0–2 showed a remarkable evolution with overlaps of progression and downgrade within 3 weeks after symptom onset, and mostly remained the same thereafter. Pattern 3 and 4 showed a remarkable evolution (progression or downgrade) within 2 weeks, and most of them remained afterwards (**Figure 2**).

Figures 3–6 presented CT findings with disease progression in Pattern 1 to 4 cases. Pattern 1 and 2 showed limited progression with increasing density and size of lesions from 1 to 2 weeks after onset, while had complete absorption subsequently. Pattern 3 showed a fast progression from patchy GGO to extensively mixed GGO and consolidation within 2 weeks, and subsequently turned into mixed GGO and linear opacities. Pattern 4 showed a considerably fast progression to diffusely mixed consolidation and interlobular septal thickening in both lungs and had adverse outcome within 1 week.

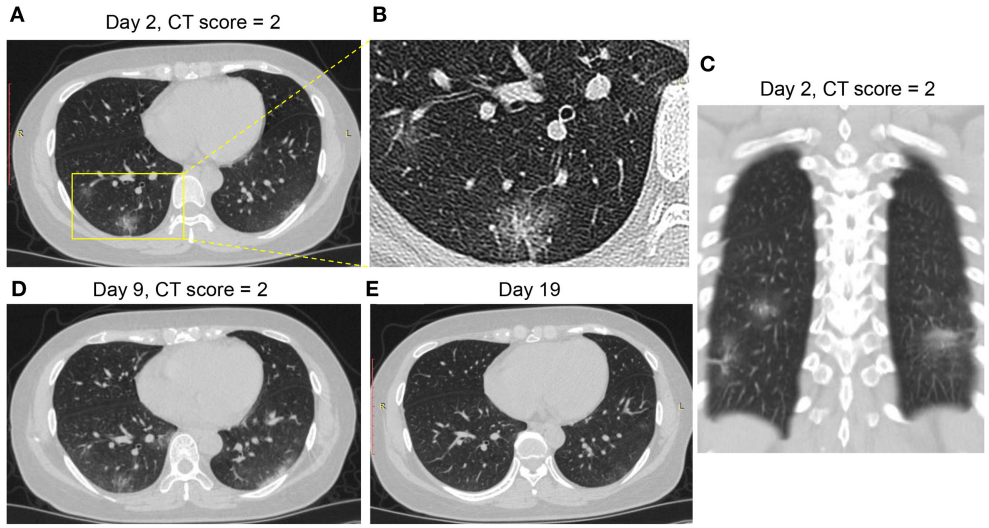


FIGURE 3 | CT Pattern 1 (bronchopneumonia pattern) in a 38-year-old woman with COVID-19 pneumonia who was admitted to hospital at day 2 after symptom onset. **(A–C)** Axial and coronal CT images demonstrate multifocal peribronchial ground-glass opacity (GGO) at day 2; Axial CT images demonstrate increasing density and size of lesions at day 9 **(D)** and subsequently complete absorption at day 19 **(E)**.

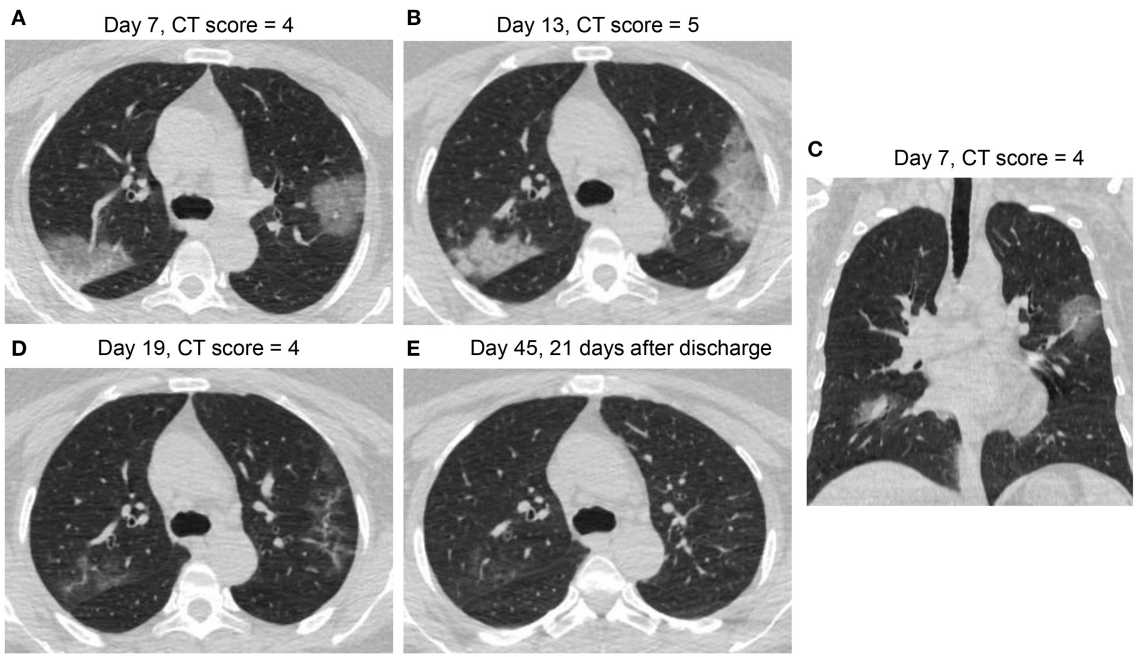


FIGURE 4 | CT Pattern 2 (organizing pneumonia pattern) in a 49-year-old woman with COVID-19 pneumonia who was admitted to hospital at day 7 after symptom onset and discharged at day 24. **(A,C)** Axial and coronal CT images demonstrate multifocal ground-glass opacity (GGO), mixed GGO and consolidation at day 7; Axial CT images demonstrate consolidation at day 13 **(B)**, subsequent absorption with mixed GGO and linear opacities at day 19 **(D)**, and complete absorption at day 45 **(E)**.

Prognostic Significance of Pneumonic CT Pattern in COVID-19

Supplementary Table 1 detailed the clinical, laboratory and CT imaging characteristics of patients in clinical outcome and pulmonary sequelae on CT. Significant differences between discharge and adverse outcome were found in age, disease severity, comorbidity, laboratory results, CT pattern and CT score (all $P < 0.05$). For pulmonary sequelae, significant

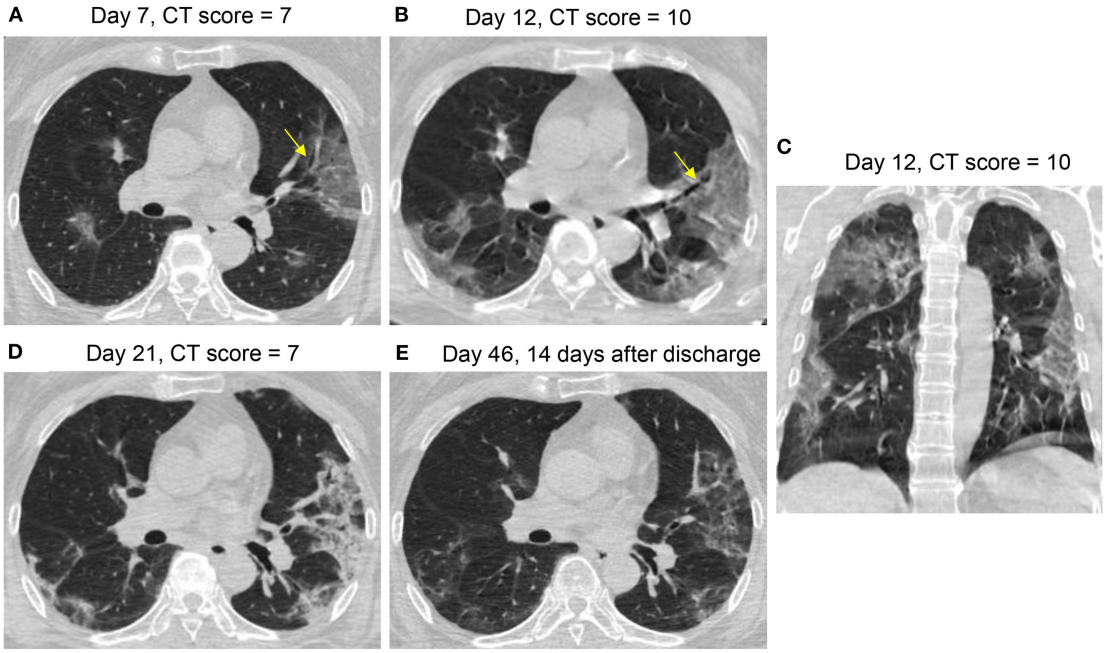


FIGURE 5 | CT Pattern 3 (progressive organizing pneumonia pattern) in a 65-year-old woman with COVID-19 pneumonia who was admitted to hospital at day 7 after symptom onset and discharged at day 24. Axial and coronal CT images demonstrate a fast progression from patchy ground-glass opacity (GGO) with slight bronchial dilatation (arrow) at day 7 **(A)**, to extensive GGO and consolidation with progressive bronchial dilatation (arrow) at day 12 **(B,C)**; Axial CT images reveal that extensive GGO and consolidation turned into consolidation and reticulation at day 21 **(D)** and into mixed GGO and linear opacities at day 46 **(E)**.

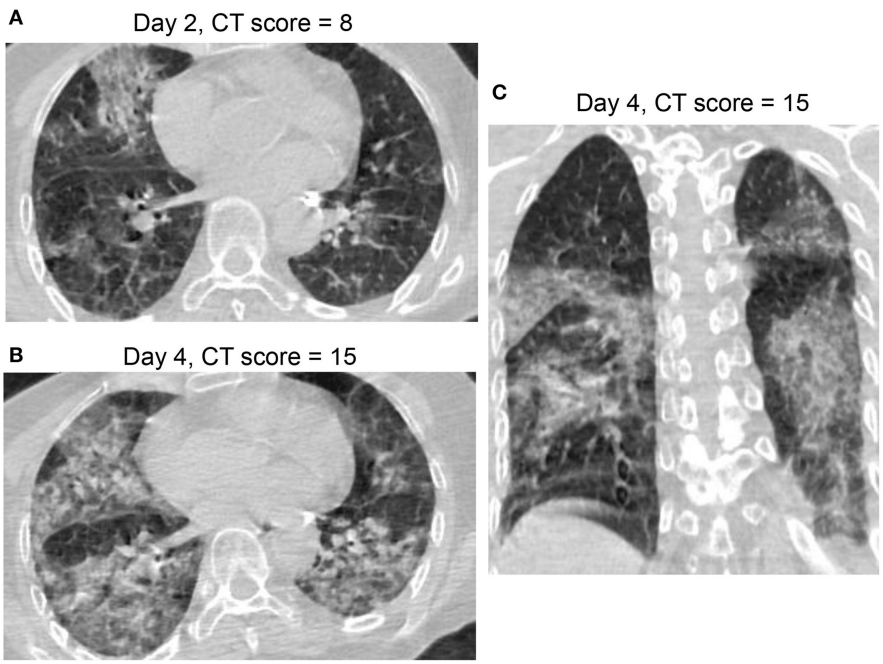


FIGURE 6 | CT Pattern 4 (diffuse alveolar damage pattern) in an 82-year-old woman COVID-19 pneumonia and with history of cardiovascular disease and chronic obstructive pulmonary disease, who was admitted to intensive care unit with mechanical ventilation at day 7 after symptom onset and died at day 39. Axial CT images demonstrate a fast progression from mixed ground-glass opacity (GGO) and consolidation at day 2 **(A)** to a geographic distribution of mixed consolidation and interlobular septal thickening at day 4 **(B)**; **(C)** Coronal CT image demonstrates mixed consolidation and interlobular septal thickening with diffused distribution of both lungs.

TABLE 3 | Risk factors associated with adverse outcome in patients with COVID-19 pneumonia.

Variable	Stratification	Univariate analysis			Multivariate analysis		
		HR	95% CI	*P*-value	HR	95% CI	*P*-value
Age (years)	≥65 vs. < 65 (Ref.)	9.39	2.38–37.11	**0.001**	3.04	0.74–12.56	0.124
Sex	Male vs. female (Ref.)	0.86	0.27–2.77	0.805			
Comorbidity	Yes vs. No (Ref.)	4.14	1.09–15.71	**0.037**			
Disease severity	Severe, critical illness vs. Mild (Ref.)	4.62	2.04–10.46	**< 0.001**			
Laboratory test at admission							
Lymphocyte percentage (%)	< 20 vs. ≥20 (Ref.)	1.00	0.24–4.16	0.998			
Monocyte percentage (%)	>10 vs. ≤10 (Ref.)	0.33	0.04–2.60	0.294			
Leukocyte count (10^9/L)	< 3.5 vs. ≥3.5 (Ref.)	0.03	0–76.60	0.390			
Alanine Aminotransferase (U/L)	>50 vs. ≤50 (Ref.)	0.82	0.21–3.16	0.820			
Aspartate Aminotransferase (U/L)	>40 vs. ≤40 (Ref.)	2.01	0.63–6.40	0.239			
Creatine kinase (U/L)	>310 vs. ≤310 (Ref.)	3.39	0.87–13.18	0.078			
Neutrophil percentage (%)	>75 vs. ≤75 (Ref.)	14.12	1.75–114.21	**0.013**			
C-reactive protein (mg/L)	>10 vs. ≤10 (Ref.)	53.87	0.12–2.5 × 10^4	0.203			
Hemoglobin (g/L)	< 130 vs. ≥130 (Ref.)	0.69	0.17–2.83	0.606			
CT findings							
GGO only	Yes vs. No (Ref.)	2.79	0.34–23.19	0.343			
Consolidation	Yes vs. No (Ref.)	0.04	0–6781	0.607			
GGO and consolidation	Yes vs. No (Ref.)	3.24	0.93–11.27	0.065			
Linear opacity	Yes vs. No (Ref.)	—-	—-	—-			
GGO and linear opacity	Yes vs. No (Ref.)	0.04	0–2.3 × 10^4	0.641			
Consolidation and linear opacity	Yes vs. No (Ref.)	0.05	0–1.7 × 10^6	0.730			
Three mixed signs	Yes vs. No (Ref.)	0.47	0.13–1.74	0.255			
Number of lobe affected	>3 vs. ≤3 (Ref.)	4.86	0.59–39.77	0.141			
CT severity score	≥10 vs. < 10 (Ref.)	11.66	2.31–58.75	**0.003**			
CT pattern	Pattern 4 vs. Pattern 0–3 (Ref.)	36.67	4.38–307.25	**0.001**	18.90	1.91–186.60	**0.012**

Ref. refers to the stratification of variable as reference in the Cox hazard-proportional regression analysis.
HR, hazard ratio; 95% CI, 95% confidence interval; GGO, ground glass opacity; Three mixed signs, GGO, consolidation and linear opacity; Pattern 0, negative; Pattern 1, organizing pneumonia pattern; Pattern 2, progressive organizing pneumonia pattern; Pattern 4, diffuse alveolar damage pattern. The bold value refers to P < 0.05.

differences between complete absorption and residuals were found in age, elevated neutrophil percentage, elevated C-reactive protein, CT pattern and CT score (all $P < 0.05$).

Correlations of CT Pattern With Clinical Outcomes

Univariate Cox proportional-hazards regression indicated that CT Pattern 4 [Hazard ratio [HR] 36.67, 95% confidence interval [95% CI] 4.38–307.25, $P = 0.001$] significantly correlated with adverse outcomes. Besides, age ≥65 years (HR 9.39, 95% CI 2.38–37.11, $P = 0.001$), comorbidity (HR 4.14, 95% CI 1.09–15.71, $P = 0.037$), severe or critical illness (HR 4.62, 95% CI 2.04–10.46, $P < 0.001$), presence of fatigue (HR 3.62, 95% CI 1.16–11.28, $P = 0.027$) and chest congestion and/or shortness of breath (HR 3.81, 95% CI 1.19–12.18, $P = 0.024$), neutrophil percentage >75% (HR 14.12, 95% CI 1.75–114.21, $P = 0.013$), CT score ≥10 (HR 11.66, 95% CI 2.31–58.75, $P = 0.003$) were associated with adverse outcomes (**Table 3**). Multivariate analysis indicated that after controlling for age, Pattern 4 was found to be an independent risk factor for adverse outcomes (HR 18.90, 95% CI 1.91–186.60, $P = 0.012$) (**Figure 7**).

Correlations of CT Pattern With Pulmonary Sequelae on CT After Discharge

By univariate Cox proportional-hazards regression, it was found that CT Pattern 3 or 4 (HR 0.23, 95% CI 0.07–0.78, $P = 0.017$) were significantly related with pulmonary sequelae. Beyond, significant factors included age ≥45 years (HR 0.36, 95% CI 0.15–0.88, $P = 0.025$), C-reactive protein concentration >10 mg/L (HR 0.28, 95% CI 0.12–0.65, $P = 0.003$), number of lobe affected >3 (HR 0.34, 95% CI 0.16–0.71, $P = 0.005$), CT score ≥4 (HR 0.32, 95% CI 0.15–0.65, $P = 0.002$) (**Table 4**). The multivariate analysis showed that Pattern 3 or 4 (HR 0.26, 95% CI 0.08–0.88, $P = 0.030$) and C-reactive protein (HR 0.31, 95% CI 0.13–0.72, $P = 0.006$) were two independent factors associated with pulmonary residuals (**Figure 8**).

DISCUSSION

By delineating the COVID-19 pneumonic CT patterns and their evolutional characteristics, this study aimed to determine their value in predicting adverse outcomes. Results indicated that CT Pattern 4 was associated with a higher rate of an adverse outcome

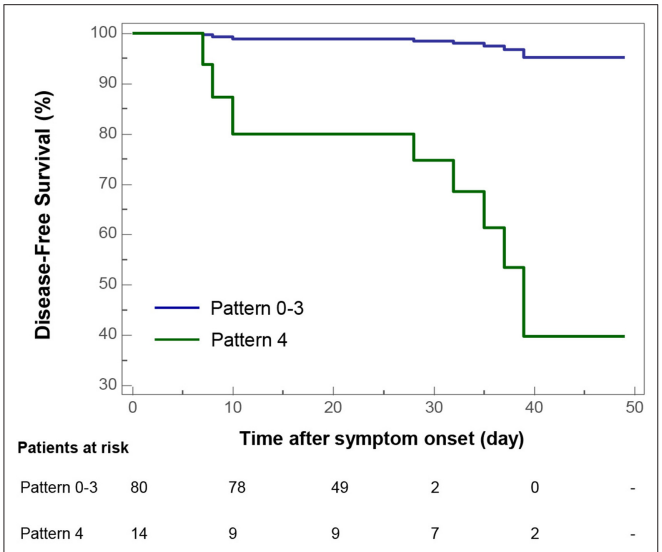


FIGURE 7 | Kaplan-Meier curve plots showing time from symptom onset to adverse outcome events (admission to intensive care unit, use of mechanical ventilation, or death) by categories of COVID-19 pneumonic CT pattern (Pattern 4 vs. Pattern 0–3 as reference).

after controlling for age; meanwhile, Pattern 3 and 4 showed more prevalence of pulmonary residuals on CT. Individual CT pattern for prognostic implication can be determined within 2 weeks after symptom onset due to the remarkable evolution of patterns before 2 weeks and subsequent stabilization or evolution without prognostic impacts.

Three kinds of phenotypes by characterizing the hypoxemia-related severity have been proposed to guide the respiratory treatment for COVID-19 (23–25). Among them, a two-phenotype of type L (low) and H (high) and a five-phenotype were defined to delineate the disease severity, mainly for hypoxemia state by clinical and/or imaging findings (23, 25). While, another three-phenotype stemmed from CT findings (multiple, focal, possibly overperfused GGO; inhomogeneously distributed atelectasis; a patchy, ARDS-like pattern) (24). These phenotype classifications could be supplement to Diagnosis and Treatment Protocol for Novel Coronavirus Pneumonia (Trial Version 7) (21). By comparison, our CT pattern categorization detailed the extent of lung injury in COVID-19. Among them, Pattern 2 to 4 showed compatible with CT signs of three-phenotype (24). Pattern 1 was found to be linked with a good prognosis as well as Pattern 0. This resembled the prior reports of H1N1 pneumonia (17). Pathologically, organization has been recognized as a common response in lung injury (15, 26). In this study, OP patterns accounted for 60% and the overall degree of lung injury especially for Pattern 2 was mild where reparative process and resolution of lesions seem to follow. Note that more prevalence of residuals may indicate a protracted disease course in Pattern 3. This may be related to older patients with comorbidity and decreased lymphocyte percentage. For Pattern 4, 85.7% cases had an adverse outcome. Pathologically, intraalveolar edema, fibrin, and variable cellular infiltrates with a hyaline membrane were observed in DAD (16, 27). It may be more severe disease, more prevalence of elevated creatine kinase, neutrophil percentage and C-reactive protein that led to the higher rate of adverse outcomes in Pattern 4. Previous studies have demonstrated the residual fibrosis in 38 and 85% of DAD survivals, which may be related to barotrauma due to mechanical ventilation or oxygen toxicity (28). Differently, fibrosis was not pathologically observed in COVID-19 death perhaps due to the short disease course of 15 days from onset to death (29). A long-term follow up of discharged DAD patients who survived after mechanical ventilation or continuous high-flow oxygen therapy would be required to further understand the sequelae.

Diverse evolutions with overlaps of progression and downgrading were found in Pattern 0–2 within 3 weeks and Pattern 3–4 within 2 weeks after onset. Most of them remained thereafter. It is noting that 28.6% of Pattern 1 progressed to Pattern 2 from 2 to 3 weeks. This evolution was consistent with prior report of acute and progressive characteristics of COVID-19 (11). In addition, this progression from Pattern 1 to 2 after 2 weeks may reflect the organization regarding lung repair and would have good prognosis (15). From the above, individual CT pattern for prognostic implication can be determined within 2 weeks after onset due to the remarkable evolution of patterns before 2 weeks and subsequent stabilization or evolution without prognostic impacts.

Univariate analysis indicated that age ≥65 years, presence of comorbidity (70% hypertension and diabetes mellitus), severe or critical illness, neutrophil percentage >75%, CT score ≥10, CT Pattern 4 were significantly related with adverse outcome. These findings echo the latest reports (7, 8). In details, a poor clinical outcome was associated with increased age (>65 years), presence of comorbidity as well as elevated levels of hypersensitive troponin I, leukocyte and neutrophil in COVID-19 patients (7–9). By multivariate analysis, only Pattern 4 was associated with an adverse outcome after controlling age. In our cohort, most of Pattern 4 cases were age ≥65 years (64.3%), presence of comorbidity (71.4%) and critical illness (57.1%). This may be the underlying reason regarding Pattern 4 as only significant factor in multivariate analysis. This further enhanced the potential role of CT pattern in predicting the risks of adverse outcomes in COVID-19.

As for pulmonary sequelae, CT Pattern 3 or 4 and elevated C-reactive protein were two independent factors associated with pulmonary residuals on CT. Pattern 3 and 4 showed more prevalence of pulmonary residuals than others. This may be linked with more severe CT findings of these cases with more number of lobe affected and CT scores. In concert with MERS studies that radiological sequelae can remain at least 1 year after infection (30), our study found similar but slighter residuals mainly presenting with linear opacities and/or a few consolidation and GGO. Beyond, elevated C-reactive protein may indicate the state of tissue injury and/or acute inflammation, which may suggest a risk indication of progression to a critical disease state (31). In this regard, elevated C-reactive protein may be predictive of radiological sequelae. Prior studies indicated that radiological sequelae from SARS and MERS may suggest

TABLE 4 | Risk factors associated with pulmonary sequelae of lesion resolution at 2–3 weeks after discharge in patients with COVID-19 pneumonia.

Variable	Stratification	Univariate analysis			Multivariate analysis		
		HR	95% CI	*P*-value	HR	95% CI	*P*-value
Age (yr)	≥45 vs. < 45 (Ref.)	0.36	0.15–0.88	**0.025**			
Sex	Male vs. Female (Ref.)	1.09	0.53–2.25	0.806			
Comorbidity	Yes vs. No (Ref.)	0.46	0.18–1.21	0.116			
Disease severity	Severe vs. Mild (Ref.)	0.87	0.12–6.43	0.893			
Laboratory test at admission							
Lymphocyte percentage (%)	< 20 vs. ≥20 (Ref.)	0.50	0.22–1.13	0.094			
Monocyte percentage (%)	>10 vs. ≤10 (Ref.)	1.94	0.92–4.09	0.082			
Leukocyte count (10^9/L)	< 3.5 vs. ≥3.5 (Ref.)	0.96	0.39–2.38	0.928			
Alanine Aminotransferase (U/L)	>50 vs. ≤50 (Ref.)	0.50	0.17–1.46	0.202			
Aspartate Aminotransferase (U/L)	>40 vs. ≤40 (Ref.)	0.69	0.27–1.81	0.451			
Creatine kinase (U/L)	>310 vs. ≤310 (Ref.)	0.50	0.12–2.12	0.349			
Neutrophil percentage (%)	>75 vs. ≤75 (Ref.)	0.32	0.10–1.06	0.062			
C-reactive protein (mg/L)	>10 vs. ≤10 (Ref.)	0.28	0.12–0.65	**0.003**	0.31	0.13–0.72	**0.006**
Hemoglobin (g/L)	< 130 vs. ≥130 (Ref.)	0.36	0.09–1.54	0.169			
CT findings							
GGO only	Yes vs. No (Ref.)	1.14	0.34–3.84	0.827			
Consolidation	Yes vs. No (Ref.)	2.89	1.08–7.72	**0.035**			
GGO and consolidation	Yes vs. No (Ref.)	1.02	0.45–2.28	0.969			
Linear opacity	Yes vs. No (Ref.)	–	–	–			
GGO and linear opacity	Yes vs. No (Ref.)	0.88	0.21–3.71	0.856			
Consolidation and linear opacity	Yes vs. No (Ref.)	0.89	0.12–6.59	0.911			
Three mixed signs	Yes vs. No (Ref.)	0.52	0.24–1.13	0.098			
Number of lobe affected	>3 vs. ≤3 (Ref.)	0.34	0.16–0.71	**0.005**			
CT severity score	≥4 vs. < 4 (Ref.)	0.32	0.15–0.65	**0.002**			
CT Pattern	Pattern 3,4 vs. Pattern 0–2 (Ref.)	0.23	0.07–0.77	**0.017**	0.26	0.08–0.88	**0.030**

Ref. refers to the stratification of variable as reference in the Cox hazard-proportional regression analysis.
HR, hazard ratio; 95% CI, 95% confidence interval; GGO, ground glass opacity; Three mixed signs, GGO, consolidation and linear opacity; Pattern 0, negative; Pattern 1, organizing pneumonia pattern; Pattern 2, progressive organizing pneumonia pattern; Pattern 4, diffuse alveolar damage pattern. The bold value refers to P < 0.05.

the abnormal or repaired lung function (30, 32). Despite the slight residuals in COVID-19, a long-term follow-up is required to further trace the resolution and associations with lung function.

This study had some limitations. The first was the small sample, especially for those with adverse outcomes and/or with Pattern 4. A larger sample is required to further verify the findings regarding the risk factors affecting the adverse outcome and disease progression, as well as factors in relation to respiratory treatment strategy (e.g., non-invasive or mechanical ventilation). Besides, more clinical indicators such as body mass index would be gathered to explore the potential correlations with prognosis due to the prior report of obesity as risk factor of severe COVID-19 (33). Second, because discharged patients remained during the recovery and pulmonary CT residuals were unknown at the time of our analysis, a long-term follow-up is required to further trace the outcome of lesion absorption, as well as changes in lung functions. Third, despite of using a high-resolution CT protocol recommended by American College of Radiology (34), varying CT scanners may have potential impacts on CT pattern evaluation. A large sample from these CT scanners should be collected to first clarify the impacts and thereby facilitate the generalization of our findings. Forth, multicenter data collection may lead to selective bias of patients with various CT patterns. Although no significance in univariate analysis (see more in **Supplement Material**), potential impacts from varying hospital, epicenter vs. non-epicenter should be considered in further studies. Last, given the inadequate CT resource, an alternative pattern categorization by X-ray image and/or available quick-test laboratory indicators should be further explored.

In conclusion, CT pattern categorization of COVID-19 pneumonia based on chest CT within 2 weeks after symptom onset has prognostic significance. CT pattern 4 cases present high risk of admission to ICU, need for mechanical ventilation or death, while Pattern 3 and 4 signal likelihood of pulmonary residuals on CT. In this regard, when allocating medical resources, pattern 0–2 cases could be considered as mild group and then admitted to community hospital or mobile cabin hospital, while pattern 3 or 4 should be admitted

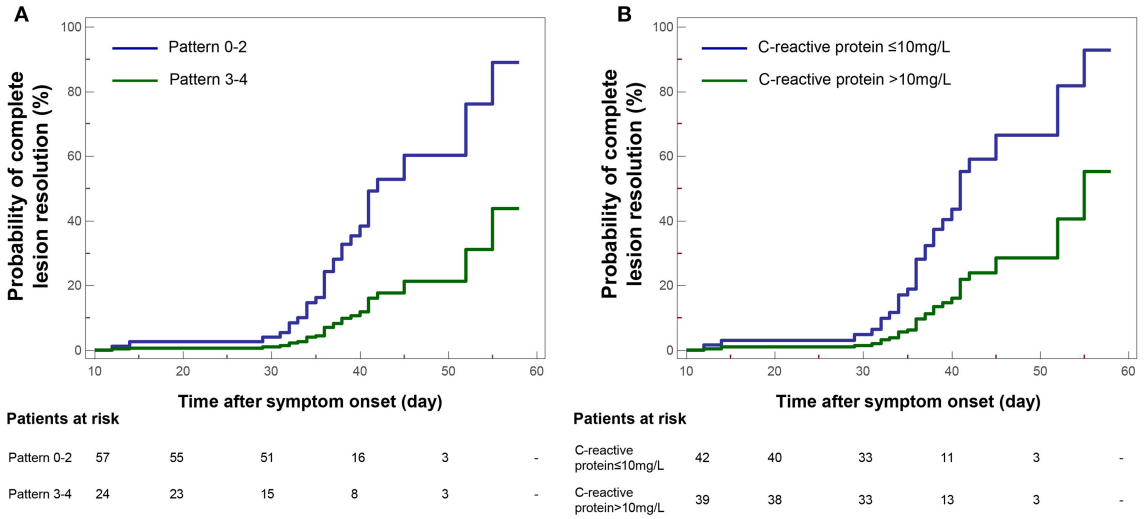


FIGURE 8 | Kaplan-Meier curve plots showing time from symptom onset to complete resolution of pulmonary lesions by **(A)** categories of COVID-19 pneumonic CT pattern (Pattern 3–4 vs. Pattern 0–2 as reference), and **(B)** conditions of C-reactive protein.

to designate general hospital. These findings would help early prognostic stratification of COVID-19 and facilitate the decision making for treatment strategy and optimal use of healthcare resources.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The internal review board of the First Affiliated Hospital of Xi'an Jiaotong University. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

CJ and JY contributed to the literature search. CT, YW, HZha, TL, ZLiu, ZJ, RL, ZW, FL, JZ, SC, YL, HL, ZLi, YL, HZho, XW, and ZR contributed to data collection and analysis. CJ, YW, and JY contributed to data interpretation. CJ, CT, CW, and JY contributed to writing of the manuscript. All authors contributed to the study conception, design, article, and approved the submitted version.

ACKNOWLEDGMENTS

We thank all the participants for their contribution in this study.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2020.567672/full#supplementary-material

REFERENCES

1. Fauci AS, Lane HC, Redfield RR. Covid-19 - navigating the uncharted. *N Engl J Med.* (2020) 382:1268–9. doi: 10.1056/NEJMe2002387
2. WHO. Coronavirus disease 2019. 2020. https://covid19.who.int/ (accessed May 29, 2020).
3. Guan WJ, Ni ZY, Hu Y, Liang WH, Ou CQ, He JX, et al. Clinical characteristics of coronavirus disease 2019 in China. *N Engl J Med.* (2020) 382:1708–20. doi: 10.1056/NEJMoa2002032
4. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet.* (2020) 395:497–506. doi: 10.1016/S0140-6736(20)30183-5
5. Wang D, Hu B, Hu C, Zhu F, Liu X, Zhang J, et al. Clinical characteristics of 138 hospitalized patients with 2019 novel coronavirus-infected pneumonia in Wuhan, China. *JAMA.* (2020) 323:1061–9. doi: 10.1001/jama.2020.1585
6. Pormohammad A, Ghorbani S, Baradaran B, Khatam A, Turner R, Mansournia MA, et al. *Clinical Characteristics, Laboratory Findings, Radiographic Signs and Outcomes of 52,251 Patients With Confirmed COVID-19 Infection: A Systematic Review and Meta-Analysis. Preprints [Preprint].* (2020) Available online at: https://www.preprints.org/manuscript/202003.0252/v1. (assessed March 18, 2020)
7. Zhou F, Yu T, Du R, Fan G, Liu Y, Liu Z, et al. Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: a retrospective cohort study. *Lancet.* (2020) 395:1054–62. doi: 10.1016/S0140-6736(20)30566-3
8. Zhao X, Zhang B, Li P, Ma C, Gu J, Hou P, et al. Incidence, clinical characteristics and prognostic factor of patients with COVID-19: a systematic review and meta-analysis. *medRxiv [Preprint].* (2020) doi: 10.1101/2020.03.17.20037572

9. Hu L, Chen S, Fu Y, Gao Z, Long H, Ren H, et al. Risk factors associated with clinical outcomes in 323 COVID-19 patients in Wuhan, China. *Clin Infect Dis.* (2020) doi: 10.1093/cid/ciaa539. [Epub ahead of print].
10. Hosseiny M, Kooraki S, Gholamrezanezhad A, Reddy S, Myers L. Radiology perspective of coronavirus xisease 2019 (COVID-19): lessons from severe acute respiratory syndrome and middle east respiratory syndrome. *AJR Am J Roentgenol.* (2020) 214:1078–82. doi: 10.2214/AJR.20.22969
11. Wang Y, Dong C, Hu Y, Li C, Ren Q, Zhang X, et al. Temporal changes of CT findings in 90 patients with COVID-19 pneumonia: a longitudinal study. *Radiology*. (2020) 296:E55–E64. doi: 10.1148/radiol.2020200843
12. Shi H, Han X, Jiang N, Cao Y, Alwalid O, Gu J, et al. Radiological findings from 81 patients with COVID-19 pneumonia in Wuhan, China: a descriptive study. *Lancet Infect Dis.* (2020) 20:25–434. doi: 10.1016/S1473-3099(20)30086-4
13. Wu J, Wu X, Zeng W, Guo D, Fang Z, Chen L, et al. Chest CT findings in patients with corona virus disease 2019 and its relationship with clinical features. *Invest Radiol.* (2020) 55:257–61. doi: 10.1097/RLI.0000000000000670
14. Salehi S, Abedi A, Balakrishnan S, et al. Coronavirus disease 2019 (COVID-19): a systematic review of imaging findings in 919 patients. *Am J Roentgenol.* (2020) 215:87–93. doi: 10.2214/AJR.20.23034
15. Kligerman SJ, Franks TJ, Galvin JR. From the radiologic pathology archives: organization and fibrosis as a response to lung injury in diffuse alveolar damage, organizing pneumonia, and acute fibrinous and organizing pneumonia. *Radiographics.* (2013) 33:1951–75. doi: 10.1148/rg.337130057
16. Xu Z, Shi L, Wang Y, Zhang J, Huang L, Zhang C, et al. Pathological findings of COVID-19 associated with acute respiratory distress syndrome. *Lancet Respir Med.* (2020) 8:420–2. doi: 10.1016/S2213-2600(20)30076-X
17. Kang H, Lee KS, Jeong YJ, Lee HY, Kim KI, Nam KJ. Computed tomography findings of influenza a (H1N1) pneumonia in adults: pattern analysis and prognostic comparisons. *J Comput Assist Tomogr.* (2012) 36:285–90. doi: 10.1097/RCT.0b013e31825588e6
18. Lee KS. Pneumonia associated with 2019 novel coronavirus: can computed tomographic findings help predict the prognosis of the disease? *Korean J Radiol.* (2020) 21:257–8. doi: 10.3348/kjr.2020.0096
19. Paul NS, Roberts H, Butany J, Chung, TB, Gold W, et al. Radiologic pattern of disease in patients with severe acute respiratory syndrome: the Toronto experience. *Radiographics.* (2004) 24:553–63. doi: 10.1148/rg.242035193
20. WHO. *Clinical Management of Severe Acute Respiratory Infection When Novel Coronavirus (nCoV) Infection Is Suspected.* (2020) Available online at: https://www.who.int/publications-detail /clinical-management-of-severe-acute-respiratory-infection-when-novel-coronavirus-(ncov)-infection-is-suspected (accessed March 15, 2020).
21. National Health Commission of the People's Republic of China. *Diagnosis and Treatment of Pneumonia Caused by 2019-nCoV (version 7)*. Available online at: http://www.gov.cn/zhengce/zhengceku/ 2020-03/04/content_5486705.htm (accessed March 15, 2020).
22. Chang YC, Yu CJ, Chang SC, Galvin JR, Liu HM, Hsiao CH, et al. Pulmonary sequelae in convalescent patients after severe acute respiratory syndrome: evaluation with thin-section CT. *Radiology.* (2005) 236:1067–75. doi: 10.1148/radiol.2363040958
23. Gattinoni L, Chiumello D, Caironi P, Busana M, Romitti F, Brazzi L, et al. COVID-19 pneumonia: different respiratorytreatment for different phenotypes? *Intensive Care Med.* (2020) 46:1099–102. doi: 10.1007/s00134-020-06033-2
24. Robba C, Battaglini D, Ball L, Patroniti N, Loconte M, Brunetti I, et al. Distinct phenotypes require distinct respiratory management strategies in severe COVID-19. *Respir Physiol Neurobiol.* (2020) 279:103455. doi: 10.1016/j.resp.2020.103455
25. Rello J, Storti E, Belliato M, Ricardo S. Clinical phenotypes of SARS-CoV-2: Implications for clinicians and researchers. *Eur Respir J.* (2020) 55:2001028. doi: 10.1183/13993003.01028-2020
26. Baque-Juston M, Pellegrin A, Leroy S, Marquette CH, Padovani B. Organizing pneumonia: what is it? A conceptual approach and pictorial review. *Diagn Interv Imaging.* (2014) 95:771–7. doi: 10.1016/j.diii.2014.01.004
27. Franquet T. Imaging of pulmonary viral pneumonia. *Radiology.* (2011) 260:18–39. doi: 10.1148/radiol.11092149
28. Nöbauer-Huhmann I-M, Eibenberger K, Schaefer-Prokop C, Steltzer H, Schlick W, Strasser K, et al. Changes in lung parenchyma after acute respiratory distress syndrome (ARDS): assessment with high-resolution computed tomography. *Eur Radiol.* (2001) 11:2436–43. doi: 10.1007/s003300101103
29. Liu Q, Wang RS, Qu GQ, Wang YY, Liu P, Zhu YZ, et al. Gross examination report of a COVID-19 death autopsy. *Fa Yi Xue Za Zhi*. (2020) 36:21–23. (In Chinese). doi: 10.12116/j.issn.1004-5619.2020.01.005
30. Park WB, Jun KIl, Kim G., Choi JP, Rhee JY, Cheon S, et al. Correlation between pneumonia severity and pulmonary complications in Middle East respiratory syndrome. *J Korean Med Sci.* (2018) 33:e169. doi: 10.3346/jkms.2018.33.e169
31. Clyne B, Olshaker JS. The C-reactive protein. *J Emerg Med.* (1999) 17:1019–25. doi: 10.1016/S0736-4679(99)00135-3
32. Antonio GE, Wong KT, Hui DSC, Wu A, Lee N, Yuen EHY, et al. Thin-section CT in patients with severe acute respiratory syndrome following hospital discharge: preliminary experience. *Radiology.* (2003) 228:810. doi: 10.1148/radiol.2283030726
33. Sattar N, McInnes IB, McMurray J. Obesity is a risk factor for severe COVID-19 infection: multiple potential mechanisms. *Circulation.* (2020) 142:4. doi: 10.1161/CIRCULATIONAHA.120.047659
34. American College of Radiology. *ACR-STR Practice Parameter for the Performance of High-Resolution Computed Tomography (HRCT) of the Lungs in Adults*. Available online at: https://www.acr.org/-/media/ACR/Files/Practice-Parameters/HRCT-Lungs.pdf (accessed July 15, 2020).

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 18 September 2020
doi: 10.3389/fimmu.2020.552925

Strategies for Targeting SARS CoV-2: Small Molecule Inhibitors—The Current Status

Narasimha M. Beeraka[1†], Surya P. Sadhu[2†], SubbaRao V. Madhunapantula[1,3], Rajeswara Rao Pragada[2], Andrey A. Svistunov[4], Vladimir N. Nikolenko[4,5], Liudmila M. Mikhaleva[6] and Gjumrakch Aliev[6,7,8,9*]*

[1] Department of Biochemistry, Center of Excellence in Molecular Biology and Regenerative Medicine (CEMR), JSS Academy of Higher Education & Research (JSS AHER), Mysore, India, [2] AU College of Pharmaceutical Sciences, Andhra University, Visakhapatnam, India, [3] Special Interest Group in Cancer Biology and Cancer Stem Cells (SIG-CBCSC), JSS Medical College, JSS Academy of Higher Education & Research (JSS AHER), Mysore, India, [4] I. M. Sechenov First Moscow State Medical University of the Ministry of Health of the Russian Federation (Sechenov University), Moscow, Russia, [5] Department of Normal and Topographic Anatomy, M.V. Lomonosov Moscow State University, Moscow, Russia, [6] Research Institute of Human Morphology, Moscow, Russia, [7] Sechenov First Moscow State Medical University (Sechenov University), Moscow, Russia, [8] Institute of Physiologically Active Compounds, Russian Academy of Sciences, Moscow, Russia, [9] GALLY International Research Institute, San Antonio, TX, United States

OPEN ACCESS

Edited by:
Denise L. Doolan,
James Cook University, Australia

Reviewed by:
Rong Hai,
University of California, Riverside, United States
Katie Louise Flanagan,
RMIT University, Australia

***Correspondence:**
SubbaRao V. Madhunapantula
mvsstsubbarao@jssuni.edu.in
Gjumrakch Aliev
aliev03@gmail.com;
cobalt55@gallyinternational.com

† These authors have contributed equally to this work

Specialty section:
This article was submitted to Vaccines and Molecular Therapeutics, a section of the journal Frontiers in Immunology

Received: 17 April 2020
Accepted: 18 August 2020
Published: 18 September 2020

Citation:
Beeraka NM, Sadhu SP, Madhunapantula SV, Rao Pragada R, Svistunov AA, Nikolenko VN, Mikhaleva LM and Aliev G (2020) Strategies for Targeting SARS CoV-2: Small Molecule Inhibitors—The Current Status. Front. Immunol. 11:552925. doi: 10.3389/fimmu.2020.552925

Severe Acute Respiratory Syndrome-Corona Virus-2 (SARS-CoV-2) induced Coronavirus Disease - 19 (COVID-19) cases have been increasing at an alarming rate (7.4 million positive cases as on June 11 2020), causing high mortality (4,17,956 deaths as on June 11 2020) and economic loss (a 3.2% shrink in global economy in 2020) across 212 countries globally. The clinical manifestations of this disease are pneumonia, lung injury, inflammation, and severe acute respiratory syndrome (SARS). Currently, there is no vaccine or effective pharmacological agents available for the prevention/treatment of SARS-CoV2 infections. Moreover, development of a suitable vaccine is a challenging task due to antibody-dependent enhancement (ADE) and Th-2 immunopathology, which aggravates infection with SARS-CoV-2. Furthermore, the emerging SARS-CoV-2 strain exhibits several distinct genomic and structural patterns compared to other coronavirus strains, making the development of a suitable vaccine even more difficult. Therefore, the identification of novel small molecule inhibitors (NSMIs) that can interfere with viral entry or viral propagation is of special interest and is vital in managing already infected cases. SARS-CoV-2 infection is mediated by the binding of viral Spike proteins (S-protein) to human cells through a 2-step process, which involves Angiotensin Converting Enzyme-2 (ACE2) and Transmembrane Serine Protease (TMPRSS)-2. Therefore, the development of novel inhibitors of ACE2/TMPRSS2 is likely to be beneficial in combating SARS-CoV-2 infections. However, the usage of ACE-2 inhibitors to block the SARS-CoV-2 viral entry requires additional studies as there are conflicting findings and severe health complications reported for these inhibitors in patients. Hence, the current interest is shifted toward the development of NSMIs, which includes natural antiviral phytochemicals

and Nrf-2 activators to manage a SARS-CoV-2 infection. It is imperative to investigate the efficacy of existing antiviral phytochemicals and Nrf-2 activators to mitigate the SARS-CoV-2-mediated oxidative stress. Therefore, in this review, we have reviewed structural features of SARS-CoV-2 with special emphasis on key molecular targets and their known modulators that can be considered for the development of NSMIs.

Keywords: SARS-CoV, SARS-CoV-2, COVID-19, natural Nrf-2 modulators, NSMIs

INTRODUCTION

Global Burden of COVID-19

COVID-19 is a devastating disease caused by a coronavirus related to the one that caused outbreaks of Severe Acute Respiratory Syndrome (SARS) in the year 2002 (1, 2). Middle East Respiratory Syndrome (MERS)-related coronavirus is an infamous member of this cohort. COVID-19, which is caused by the SARS-CoV-2 infection, was detected in Wuhan, China in December 2019. The World Health Organization (WHO) declared this infection a pandemic on March 11 2020 due to its severity and rapid spread across the globe. As of June 11 2020, SARS-CoV-2 had infected 7.4 million individuals, and caused 4,17,956 deaths across 212 countries worldwide (**Table 1**).

Structural Features of SARS-CoV-2

Coronaviruses (CoV) belongs to a family of single-stranded RNA viruses (+RNA) that can infect a variety of mammals such as bats and humans (3). SARS-CoV-2 contains RNA of 29,891-nucleotide length, which codes for 9,860 amino acids (4). The RNA has a 5' cap and 3' poly-A tail and produces a poly-protein 1a/1ab (pp1a/pp1ab) in the host (4). SARS-CoV-2 belongs to beta CoV category and appears in a crown shape with a size of ∼60–140 nm (**Figure 1**).

Gene sequencing data revealed that SARS-CoV-2 has 89 and 82% sequence similarity with bat SARS-like-CoV-ZXC21 and human SARS-CoV, respectively (4, 5). The spike (S) protein-coding gene mutation in the *nsp2 and nsp3* regions results in the replacement of glycine (G) with serine (S) at 723 position (G723S), and an isoleucine (I) replaced with proline (P) at 1010 amino acid position (I1010P). Due to these mutations, the invading potential of SARS-CoV-2 has increased significantly toward host tissues. This virus can also be transmitted through the respiratory droplets from coughs and sneezes of infected individuals (4). This mode of aerosol transmission is possible, especially, when protracted exposure occurs in closed areas (4). The incubation time of the virus varies significantly from individual to individual. In general it takes about 6 days from the day of infection to the first appearance of symptoms. However, in a few cases the symptoms may appear only after 2 weeks (6).

Abbreviations: RTIs, Respiratory tract infections; ORF, Open reading frame; TMPRSS2, Transmembrane serine protease; ADAM17, Disintegrin and metallopeptinase-containing domain 17; SARS-CoV, Severe respiratory syndrome-coronavirus; SARS-CoV-2, Severe respiratory syndrome-Coronavirus-2 (COVID-19); GM-CSF: Granulocyte–macrophage colony-stimulating factor.

SARS-CoV2 Infection and Pulmonary Pathogenesis

Members of *Coronaviridae* are known to induce respiratory complications in humans (7, 8). At first, SARS-CoV, MERS-CoV, and SARS-CoV-2 varieties were transmitted from animals to humans which triggered severe respiratory diseases (9–11). However, subsequent transmission occurred among humans primarily due to physical contact. Hence, conventional preventive measures such as physical isolation were implemented to avoid propagation of early infection across the human population (1, 12). Similar to the SARS-CoV, the pathological manifestations of SARS-CoV-2 could induce lung malfunction in humans as indicated by the severe acute respiratory syndrome and pneumonia (12). Recent studies reported that SARS-CoV-2 infection can induce mild, moderate, and severe illness in infected patients (4). Clinical manifestations of this infection include chronic pneumonia, sepsis, septic shock, fever, and dry cough (4). A progressive respiratory failure during this infection may lead to sudden death (4). Mild illness resulting from a SARS-CoV-2 infection is characterized by the presence of malaise, headache, low fever and dyspnea. In the case of moderate illness from SARS-CoV-2, the complication is manifested by the presence of cough and mild pneumonia. Severe illness from SARS-CoV-2 is associated with chronic pneumonia, cough, SARS, hypoxia, and tachypnea (in children) followed by respiratory, and cardiovascular system failure

TABLE 1 | Recent statistics of SARS-CoV2 infection—Top 10 countries.

Country	Infected (in Millions) (%)[#]	Recovered (in Millions) (%)[$]	Deaths (in Thousands) (%)*
United States of America	2.064 (0.623)	0.800 (38.79)	115,115 (5.57)
Brazil	0.772 (0.363)	0.380 (49.23)	39,680 (5.13)
Russia	0.493 (0.338)	0.252 (51.20)	6,358 (1.28)
United Kingdom	0.290 (0.427)	0.135 (46.52)	41,128 (14.17)
Spain	0.289 (0.618)	Not available	27,136 (9.37)
India	0.287 (0.020)	0.140 (49.09)	8,107 (2.82)
Italy	0.235 (0.389)	0.169 (72.08)	34,114 (14.46)
Peru	0.208 (0.633)	0.098 (46.94)	5,903 (2.82)
Germany	0.186 (0.22)	0.170 (91.34)	8,844 (4.73)
Iran	0.177 (0.212)	0.140 (79.01)	8,506 (4.78)

[#] *Percentage of total population.*
[$] *Percentage of total infected cases.*
**Percentage of total infected cases.*
List of top 10 countries in the world affected with COVID-19.

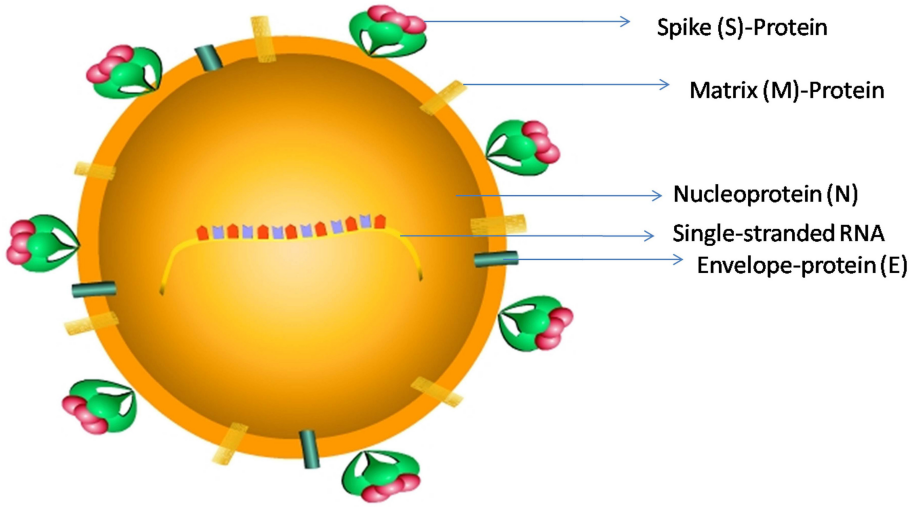


FIGURE 1 | The schematic representation of SARS-CoV-2 structure: SARS-CoV-2 has a size ranging from 60 to 140 nm, and is a spherical to elliptical shaped virus with a crown-like appearance; it consists of a single-stranded RNA genome, a Spike protein (S), a Matrix protein (M), a nucleoprotein (N), and an Envelope-protein (E).

(4). The autopsy and biopsy reports of SARS-CoV-2 patients revealed severe edema with pulmonary tissue exudates, focal reactive hyperplasia, damage to pneumocytes as well as alveolar macrophages, and patchy cellular infiltration (13).

Coronavirus-induced lung damage has been demonstrated experimentally by several investigators in animal models (14). For instance, the Sialodacryoadenitis virus and Parker's RCoV were shown to induce damage to alveolar type-I cells through the expression of pro-inflammatory cytokines, and chemokines such as *CINC-2, CINC-3, LIX, MIP-3α, and fractalkines* (15–21). For example, fractalkine promotes the infiltration of cytotoxic lymphocytes in the alveolar epithelium thereby inducing a severe inflammatory response (15, 22). Similarly, MIP-3α confers the chemotaxis of immune cells via IL-1β and TNF-α inflammatory mediators (17, 22–25). Therefore, these animal models could be used to develop effective pharmacological agents against SARS-CoV-2 infections.

Molecular Mechanisms of SARS-CoV-2 Infection

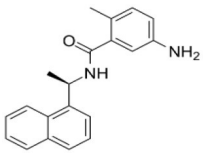
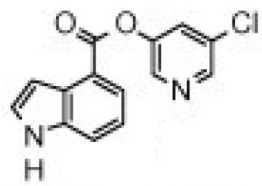
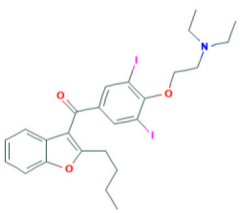
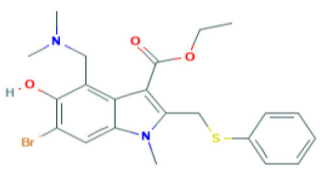
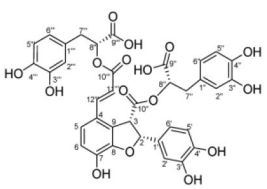
Studies from several laboratories have demonstrated that the entry of SARS-CoV-2 into human cells is facilitated by ACE-2 (26). ACE-2 is a member of the Renin-angiotensin system (RAS), which plays a vital role in cardiovascular and renal homeostasis. ACE-2 and *TMPRSS2* facilitates the entry of the virus into host cells during SARS-CoV-2 infection (7). In addition, there are other proteases such as aminopeptidase N (APN) which plays a prominent role for the entry of HCoV-NL63 and HCoV-229E into host cells (27–30). APN is a membrane-bound glycoprotein that mediates the zinc-dependent protease activity during the entry and or replication of coronavirus strains into host cells (29, 31, 32). Hence, the ACE-2 receptor's down-modulation may prevent SARS-CoV-2 viral entry/replication (33). The S-protein of SARS-CoV and other coronavirus strains are different in their structural and functional domains (3). S-protein can bind to the N-terminus of ACE-2 receptors on the outer surface of host cells including respiratory epithelium of the lungs (34–36). Identifying the key amino acid residues in S-protein of the SARS-CoV-2 strain may benefit virologists and medical scientists to develop better therapeutic agents. However, to date these details are not known, hence, there is an immediate requirement to identify the amino acids involved in binding S-proteins to ACE-2 receptors on host cell surfaces. Furthermore, investigations should also focus on establishing the structural similarities of S-protein motifs that are interacting with the ACE-2 receptors of other coronavirus strains (37–41). These investigations might help in deciphering molecular strategies to target receptor binding sites of ACE-2 proteins with SARS-CoV-2 using novel therapeutics and vaccines to avoid membrane fusion process and viral entry (7).

The TMPRSS2 protease can foster the entry of the SARS-CoV-2 virus by activating the S-protein for virus-host cell membrane fusion, consequently enhancing viral replication in the host cells (7, 42–46). TMPRSS2 plays a vital role in generating inflammatory cytokines and chemokines in lung epithelial cells by cleaving S-protein during coronavirus infections including SARS-CoV-2. Hence, TMPRSS2 is another potential therapeutic target to consider for the novel drug development against SARS-CoV-2 (46–48).

Novel Small Molecule Inhibitors (NSMIs) in the Prevention and Treatment of SARS-CoV-2 Infections

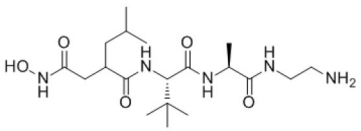
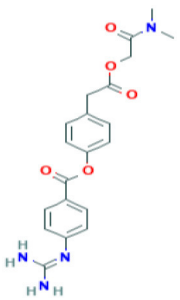
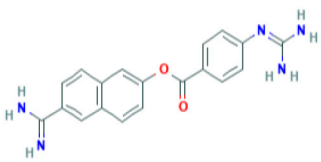
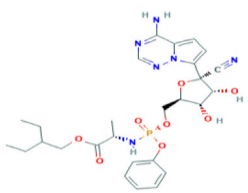
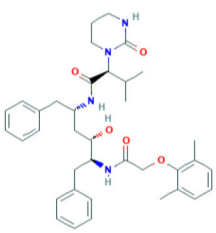
Prevention and treatment of SARS-CoV-2 infections are achieved at different levels (49). The primary approach involves physical isolation to prevent the spread of virus from individual to individual; the second approach involves inhibiting the entry of virus into human cells and the third method includes treating the infected individuals to minimize inflammatory reactions and

TABLE 2 | Structure and probable mechanism of action of NSMIs against SARS-CoV-2.

Small Molecule Inhibitors *Predicted* to be effective against SARS-CoV-2	Mechanism of Action	Structure	References
SiRNA	Targets Orf7a required for viral assembly (or) Targets Orf7b (or) Targets Orf3a required for viral budding and release Note: SiRNA is yet to be examined against SARS-CoV-2 infection	–	(50)
GRL0617	Targets non-structural proteins nsp3 (Papain like proteinase) Note: Yet to be examined against SARS-CoV-2 infection		(51)
Benzodioxolane derivatives	Targets non-structural proteins nsp3 (Papain like proteinase) in coronavirus Note: Yet to be examined against SARS-CoV-2 infection	1-[(R)-1-(1-Naphthyl)ethyl]-4-[3,4-(methylenedioxy) benzylamino] carbonylpiperidine 1-[(S)-1-(1-naphthyl) ethyl]-4-[3,4-(methylenedioxy)benzylamino] carbonylpiperidine	(52)
5-chloropyridinyl indolecarboxylate	Targets non-structural proteins nsp5 (3C-like main protease in SARS coronavirus) required for replicase synthesis Note: Yet to be examined against SARS-CoV-2 infection		(53)
2978/10 humanized antibodies	Mitigate SARS-CoV infection by targeting virus-neutralizing epitopes	–	(54)
Amiodarone	Targets SARS-CoV by inhibiting endosomal processing in host cells Note: Clinical Trials are at Recruiting Stage to test against SARS-CoV-2 infection—NCT04351763	 *(Pubchem)*	(55)
Arbidol	Targets S-protein of SARS-CoV and prevent viral fusion Note: Clinical Trials are at Recruiting Stage to test against SARS-CoV-2 infection -NCT04255017	 *(Pubchem)*	(56)
TSL-1	Targets SARS-CoV replication Note: Yet to be examined against SARS-CoV-2 infection		(57) (58)

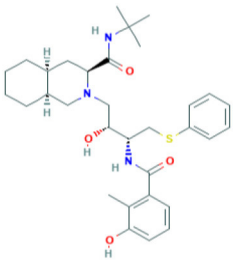
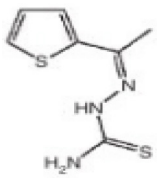
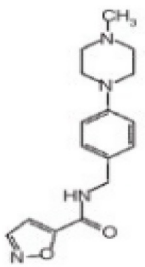
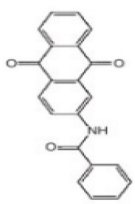
(Continued)

TABLE 2 | Continued

Small Molecule Inhibitors *Predicted* to be effective against SARS-CoV-2	Mechanism of Action	Structure	References
TACE inhibitor (TAPI-2)	Blocks SARS-CoV replication in lungs Blocks ACE2 shedding Note: Yet to be examined against SARS-CoV-2 infection		(59)
IFN—α B/D	Blocks SARS-CoV replication in lungs		(60)
IFN-β and-γ	Blocks SARS-CoV replication in lungs Note: Completed Clinical Trials for Interferon Beta-1A and Interferon Beta-1B -NCT04343768 Clinical Trials are at Recruiting Stage to test against SARS-CoV-2 infection-NCT04324463; NCT04350281 (IFN-β)		(61) (62) (63)
Camostat	TMPRSS2 serine protease Inhibitor in SARS-CoV-2 infection Note: Clinical Trials are at Recruiting stage to test against SARS-CoV-2 infection—NCT04321096	 *(Pubchem)*	(7)
Nafamostat	TMPRSS2 serine protease Inhibitor in SARS-CoV-2 virus Note: Yet to be examined against SARS-CoV-2 infection in clinical trials	 *(Pubchem)*	https://www.eurekalert.org/pub_releases/2020-03/tiom-nie032420.php
Pegylated IFN-α	Blocks SARS-CoV replication in lungs Note: Yet to be examined against SARS-CoV-2 infection	–	(2)
Remdesivir	Effective against SARS-CoV-2 infection *in vitro* Note: Clinical Trials—Recruiting stage to test against SARS-CoV-2 infection - NCT04365725	 *(Pubchem)*	(64)
Lopinavir	Predicted to block SARS-CoV-2 M^{pro} (Molecular docking studies) Note: Clinical Trials are at Recruiting stage to test against SARS-CoV-2 infection-NCT04364022	 *(Pubchem)*	(65)

(Continued)

TABLE 2 | Continued

Small Molecule Inhibitors *Predicted* to be effective against SARS-CoV-2	Mechanism of Action	Structure	References
Nelfinavir	Predicted to block SARS-CoV-2 M^{pro} (Molecular docking studies)	 *(Pubchem)*	(66)
Tocilizumab	Block SARS-CoV-2 viral induced cytokine storm—IL-6 receptor-targeted monoclonal antibody (mAb) (Ongoing clinical trials in China and Italy)—ChiCTR2000029765; NCT04377750; NCT04377659	–	*doi.org/10.1038/s41577-020-0308-3*
SSAA09E1 [[(Z)-1-thiophen-2-ylethylideneamino]thiourea]	Blocks cathepsin L required for SARS-CoV processing Note: Yet to be examined against SARS-CoV-2 infection		(67)
SSAA09E2 N-[[4-(4-methylpiperazin-1-yl)phenyl]methyl]-1,2-oxazole-5-carboxamide	Blocks SARS-CoV interaction with ACE-2 Note: Yet to be examined against SARS-CoV-2 infection		(67)
SSAA09E3 [N-(9,10-dioxo-9,10-dihydroanthracen-2-yl)benzamide]	Blocks SARS-CoV fusion to host cell membrane Note: Yet to be examined against SARS-CoV-2 infection		(67)

NCT numbers were obtained from https://clinicaltrials.gov/.

pulmonary damage. Although physical isolation is the ideal way of limiting the spread, in reality this approach is difficult to execute, hence, many pharmacological companies are actively involved in developing small molecule inhibitors to prevent the entry of the virus into human hosts (7, 49). In this regard several NSMIs have been investigated to treat SARS-CoV; but, significant breakthroughs are yet to come for treating SARS-CoV-2 (48) (**Table 2**).

Viral Entry Inhibitors vs. SARS-CoV-2

Adedeji et al. (49) reported the discovery and characterization of novel inhibitors to block SARS-CoV replication via different mechanisms. One mechanism uses screening of small molecule inhibitors using "*HIV-1 pseudotyped with SARS-CoV surface glycoprotein S (SARS-S)*" (49, 68). "SSAA09E2" is a novel small molecule inhibitor, which blocks the interaction of CoV SARS-S with ACE-2 receptors, thus blocking the viral entry (49). Another NSMI is "SSAA09E1" reported to be involved in blocking the cathepsin L, which is required for CoV-SARS-S processing to mediate viral entry into the host cell (49). SSAA09E3 is another NSMI, which can block the fusion of viral membranes with host cell surfaces (49) (**Figure 2**). Since the pathological aspects and genomic similarity of SARS-CoV-2 virus with SARS-CoV, the above strategies of inhibition may

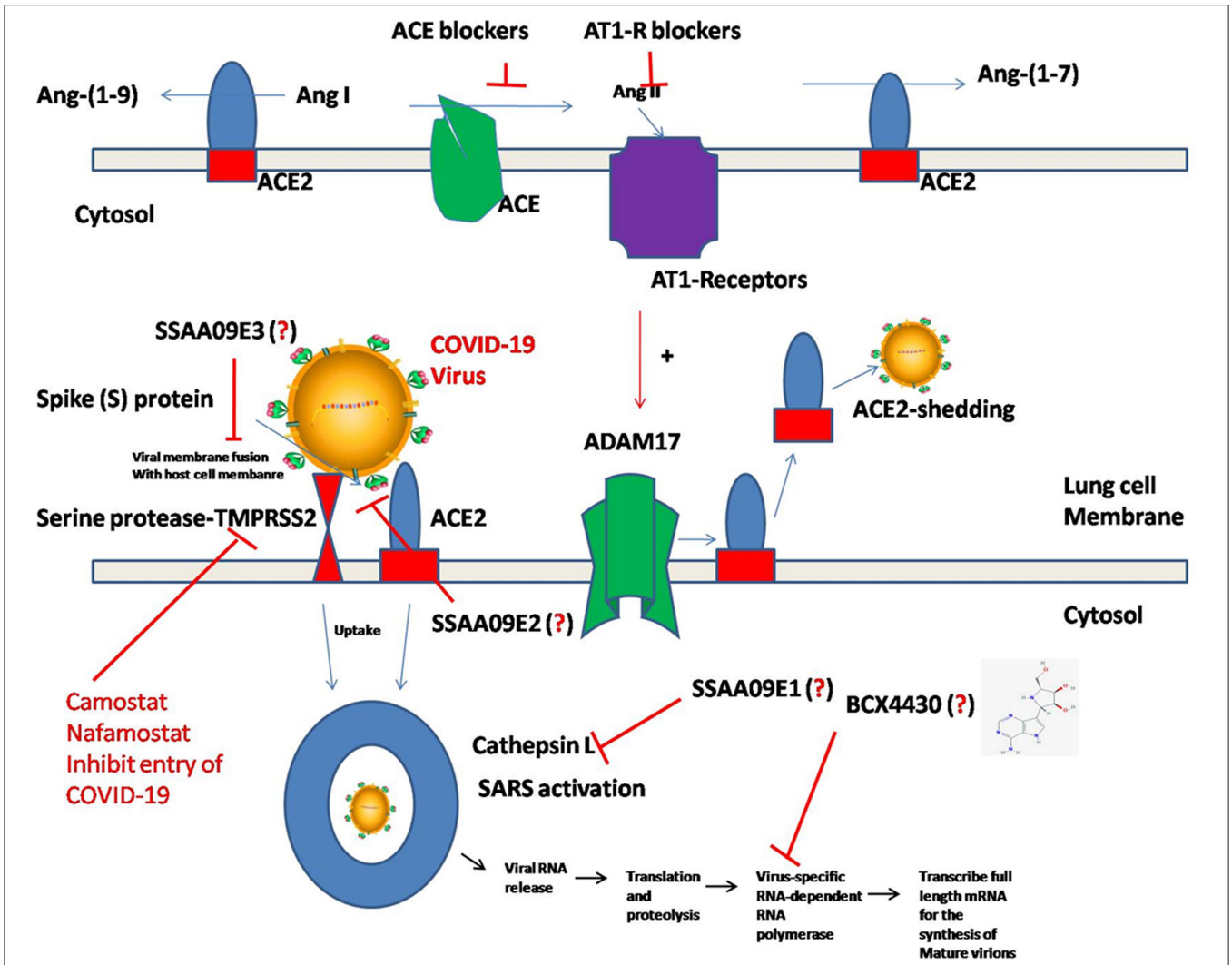


FIGURE 2 | Molecular pathogenesis of SARS-CoV-2 in human lung cells. Binding of S-protein of SARS-CoV-2 to the ACE-2 receptors triggers the processing of ACE-2 through ADAM-17/TNF-α-converting enzyme and induces the "ACE-2 shedding" into the extracellular space and facilitates uptake of SARS-CoV-2 followed by the development of SARS. Alternatively, the entry of SARS-CoV-2 by membrane TMPRSS2 serine protease'/HAT (Human Airway Trypsin-like protease)-mediated cleavage of ACE2 can facilitate SARS-CoV S-glycoprotein-mediated virus entry. Even though, several NSMIs targeting these processes were described and their mode of action against coronavirus were delineated, their efficacy against SARS-CoV-2 is yet to be tested.

be considered for developing potent pharmacological agents to prevent SARS-CoV-2 infections (49). However, the prospective research should address the efficacy of these inhibitors against SARS-CoV-2 infections.

Kinase Inhibitors vs. SARS-CoV-2

Cytokine storm was predominantly reported during SARS-CoV-2 infection. Targeting cytokine-mediated inflammatory responses induced by SARS-CoV-2 is another viable approach for mitigating the complications of viral infection. In this regard, Chang et al. (35), documented the inflammatory cascades mediated through intracellular signaling pathways conferred by the SARS-CoV in both lung epithelial cells and fibroblasts. Authors of this study have reported that S-protein of SARS-CoV efficiently mediate the IL-8 release in the infected lung cells by activating MAPKinases, and activator protein-1 (AP-1) without intervention of NF-kB cascade (35). This study suggested a promising lead for novel rational drug design through the identification of a "specific sequence motif of S-protein functional domain," which is responsible for inducing IL-8-mediated inflammatory response in lungs (35). Baricitinib is a pharmacological agent, which was reported to block the SARS-CoV-2 viral entry and inflammation through the inhibition of AP2-associated protein kinase 1 (AAK1), cyclin g-associated kinase, and janus kinase-1 and 2 (69). Chloroquine (CQ) and hydroxychloroquine (HCQ) were reported to be effective in mitigating the coronaviral load (70, 71). CQ and HCQ not only

inhibit the entry of SARS-CoV-2 but also change the pH of acidic intracellular organelles such as endosomes and lysosomes thereby preventing membrane fusion reactions. However, many contradictions and queries prevail pertaining to the use of HCQ for the treatment of COVID-19. At the time of the submission of this review, results of many clinical trials are yet to be announced, hence, the efficacy of HCQ for inhibiting SARS-CoV-2 infection is still a possibility.

Prospective studies should focus on testing the FDA approved inhibitors of "ABL-1 kinases," "PI3K/Akt/mTOR" signaling, and "MAPKinase" pathways against SARS CoV-2. Since these pathways are involved in cell survival, inflammatory cytokines production, and proliferation of cells, targeted downregulation of these pathways is likely to mitigate the exacerbations induced by coronavirus. In this direction, many of these inhibitors are currently being tested against SARS-CoV-2 (**Table 3**) (72–74). For instance, sorafenib, which inhibits RAF, is being experimented in preclinical models and early clinical trials (72). Likewise, the efficacy of IL-1 receptor antagonists and TNF-α receptor antagonists for blocking the rat coronavirus-mediated chemokine production was already proven effective in animal models (15, 75, 76). Further studies testing the safety and efficacy are warranted before considering these inhibitory agents for treating individuals infected with SARS-CoV-2 (76).

TABLE 3 | Ongoing clinical trials against SARS-CoV2 using MAPKinase Inhibitors.

MAPK Inhibitor	Mechanism of Action	References	Current Status of Clinical Trials against SARS CoV-2
Trametinib	Inhibits MAPK/ERK—kinase family proteins viz. MEK1/2 inhibitor—Inhibits MAPK/ERK—kinase family Investigational (Phase III) MEK1/ERK1/2 inhibitor	(72)	Ongoing
Selumetinib		(72)	Ongoing
Everolimus Miltefosine Teriflunamide Leflunomide	Inhibits PI3K/Akt/mTOR Kinases—Akt/mTOR Inhibits viral block of cell stress response and apoptosis	(73)	Ongoing
Dasatinib Imatinib Nilotinib	Inhibition of actin motility Blocks ABL1 kinase.	(73) (74)	Ongoing
PD98059 SB308520 SP600125	MEK inhibitor p38 inhibitor JNK inhibitor *MOA: Inhibits SARS-CoV S protein-induced IL-8 Promoter activity using above MAPK cascade inhibitors	(35)	–
Chloroquine (NCT04351724) Hydroxychlorquine (NCT04352933)	Inhibits p38 MAPK activation and blocks viral replication	(70, 71)	Ongoing

*Mechanism of action.

However, the concept of "One Drug to Treat All" should be followed to combat several devastating viral infections (77, 78). For instance, the Ebola, Marburg, and SARS-CoV-2 are undoubtedly devastating viral pathogens, which can induce high mortality as they transmit rapidly via air and body fluids (78). Outbreaks of these viruses occur sporadically and currently there are no clinically approved NSMIs available to combat these viruses. A recent report by Taylor et al. (79) demonstrated the efficacy of a synthetic adenosine analog, BCX4430 in blocking a broad spectrum of viral species viz., "coronaviruses, paramyxoviruses, and bunyaviruses" as these viruses could induce SARS, measles, and mumps. BCX4430 could efficiently block both Ebola and Marburg viral titers in non-human primate models by targeting viral RNA polymerase (78, 79). Hence, this molecule should be tested for further studies against SARS-CoV2 infections in humans.

Membrane Protease Inhibitors vs. SARS-CoV-2

Targeting the membrane protease involved in viral S-protein processing and the viral entry into host cells is another approach in mitigating SARS-CoV-2. The host cellular proteases viz., "*trypsin*,", "*miniplasmin*," "*human airway trypsin like protease*," "*tryptase Clara*," and "*TMPRSS2*" could cleave the HA glycoprotein located in influenza A virus and thereby promote viral entry into lung cells (80). The usage of serine protease inhibitors such as Camostat and Aprotinin significantly blocked the replication of influenza virus in epithelial cells of lungs and bronchioles (81). In addition, these NSMIs could block the release of inflammatory mediators such as cytokines, IL-6 and TNF-α, during this infection (81).

TMPRSS2 is a key protein involved in the pathogenesis of several seasonal viral infections including influenza, H1N1, H3N2, and H7N9 (82–85). TMPRSS2 cleaves the S-protein of coronavirus to produce unlocked, fusion-catalyzing viral forms and binds to the host cell surface thereby enhancing rapid viral entry (43, 44, 86–90). Both SARS-CoV and MERS-CoV could rapidly enter into the host cells as TMPRSS2 can facilitate viral binding to the cell surface (42, 43, 45, 87, 91, 92). TMPRSS2 also plays a vital role in the immuno-pathology of coronavirus infections including SARS-CoV-2 across lungs by inducing lung fibrosis (46). Hence, the emerging research should promote the development of NSMIs to target these proteases thereby hindering the entry of SARS-CoV-2 into host cells.

A proof-of-concept study by Iwata-Yoshikawa et al. (46) reported that SARS-CoV failed to replicate in the bronchioles and lungs of TMPRSS2 knockout mice. Authors of this study reported elevated expression of TLR3-mRNA expression in the lungs of "SARS-CoV-inoculated TMPRSS2-deficient mice" and showed enhanced TLR-3 mediated localization of dsRNA into endosomes (46). In this study, TMPRSS2 knockout has resulted in downregulation of inflammatory cytokines and chemokine expression, which are involved in the bronchiolitis obliterans organizing pneumonia (BOOP), SARS, and pulmonary fibrosis in SARS-CoV infection (46, 93, 94).

Interferon Therapy vs. SARS-CoV-2

The intricate SARS-CoV-2 pathogenesis is similar to that of SARS-CoV. Studies have reported the efficacy of IFNs to block SARS-CoV in cell line models but not against SARS-CoV-2. Among IFN- α/ -β/ and -γ, the IFN-β was reported to be the most potent blocker of SARS-CoV growth (3, 95–98). Furthermore, IFN-β and-γ have a synergistic effect in blocking SARS-CoV viral replication (62, 63). However, the effect of this combination against SARS-CoV-2 is not yet reported. Therefore, future studies should focus on determining the efficacy of IFN- α/ -β/ and –γ against SARS-CoV-2 infections.

SiRNAs vs. SARS-CoV-2

Unlike small molecule inhibitors, siRNAs are specific and can be designed to mitigate SARS-CoV *associated structural proteins* by targeting ORF4 (99, 100), ORF5 (101, 102), ORF9a (50, 103, 104), and ORF7a (50, 105–107). For example, siRNAs siSC2 and siSC5 have shown success in cultured cells as well as in preclinical mouse models in inhibiting the SARS infection without causing toxicity (108). Several other reports have also recently demonstrated the efficacy of SiRNAs to inhibit the expression of SARS-CoV genes coding for 3CL protease in cell line models (108–114). The activity of SARS-CoV 3CL protease is essential for viral replication as this protein is involved in the processing of viral proteins (114). Selective optimization and screening of hexa-chlorophene analogs can be "*active 3CL protease inhibitors*" during a SARS-CoV infection (114). Hence, the pharmacological agents/SiRNAs targeting these pathways may likely produce effective clinical outcomes in SARS-CoV-2 infections. However, clinical studies should test the utility of these agents/siRNA in reducing the burden of infections caused by SARS-CoV-2 (111).

Monoclonal Antibodies (MABs) and Other NSMIs vs. SARS-CoV-2

The genome of coronaviruses is reported to be significantly involved in coding both structural proteins, and non-structural proteins (nsp's) for the effective viral replication (115). The nsp's (nsp8C and nsp7) are required for novice CoV viral particle formation through viral ORF 1ab polyprotein processing (115). Several NSMIs were reported to target these non-structural proteins in coronavirus infections to treat SARS (115). For instance, GRL0617, a bendioxolane derivative, could target papain-like proteinases like nsp3 (51, 52, 116, 117), whereas 5-choloropyridinyl indolecarboxylate targets nsp5 (53, 118–120) and a "combination of zinc derivatives with pyrithione" targets nsp12 (121, 122); ranitidine bismuth citrate targets nsp13 (123–127). Monoclonal antibodies; CR3014 (128), mAb-201 (129), mDEF-201 (130), ampligen (131), polyICLC (61, 132), stinging nettle lectin (131), and TAPI-2 (a TACE-inhibitor) (59) are anti-coronaviral agents tested *in vivo* models of SARS. For instance, a study showed that Amiodarone (a known anti-arrhythmic agent) effectively targets coronaviral spreading in *in vitro* models (55). Working in a similar fashion, 2878/10 humanized antibodies can neutralize coronaviruses thereby reduce the complications caused by viral infections (54). However, the above NSMIs should be tested against SARS-CoV-2 viral associated proteins and against the activity of nsp's to derive an effective therapeutic intervention. Prospective research must focus on the development of novel "*helicase inhibitors, viral attachment inhibitors, and activity of Rhesus θ-defensin*" that block SARS-CoV-2 infection using *in vitro, in vivo,* and clinical studies (115). Hence, the development of NSMIs to target the synthesis of nsp's in SARS-CoV-2 may deliver cellular antiviral responses by blocking their replication in host cells (115, 133, 134).

Drug Repurposing Strategies (DRS) vs. SARS-CoV-2

Repurposing existing drugs is another strategy widely under consideration to target key proteins involved in the SARS-CoV-2 infection. In this regard, the existing NSMIs viz., antivirals (*umefenovir, remdesivir, Nitazoxanide, favipiravir, ritonavir, lopinavir, IFNs*), anticytokines, antimalaria drugs (*chloroquine, hydroxychloroquine*), and passive antibody therapies are currently being evaluated to improve clinical outcomes in SARS-CoV-2 infected patients (3, 47, 64, 135, 136). However, these agents require additional experimental and clinical validations before being tested in SARS-CoV-2 infections. For example, hydroxychloroquine (anti-malarial drug) and the tocilizumab (immunosuppressive drug) are preferred currently to mitigate viral entry and cytokine production in the SARS-CoV-2 infection. These drugs are being tested in ongoing trails in China and Italy (135, 137).

Priming the Spike (S)-protein of coronavirus by host cells using membrane proteases is a necessary process for viral entry and replication, which further determines zoonotic potential of coronaviruses (138). A recent report by Markus Hoffmann et al. (7) investigated the protease dependence of SARS-CoV-2 for its entry into cells. For example, SARS-CoV-2 uses the TMPRSS2 protease for its priming (7). Inhibition of TMPRSS2 using Camostat mesylate retarded the viral entry into Caco-2 cells (7). Camostat mesylate could be recommended as an NSMI for human clinical trials to combat the SARS-CoV-2 virus (7). This report delineated the ability of neutralizing antibody responses against S-protein to block the SARS-CoV-2 entry into host cells (139). The serum antibody responses raised to combat the "SARS-S protein/ACE-2 interface" during the SARS-CoV-2 infection indicates that the vaccination strategy may be an effective therapeutic modality against the COVID-19 infection (7).

Conflicting Reports About the ACE-2 Inhibitors Usage for Treating SARS-CoV-2 Infections

ACE-2 catalytic efficacy is significantly higher than ACE for Angiotensin-II (140). Several compounds, such as MLN-4760, were screened according to structure-based/substrate-based studies through virtual screening for inhibiting ACE-2 activity (140–143). ACE-2 is predominantly expressed in lungs, brain, heart, blood vessels, and renal organs (144, 145). ACE-2 is essential for cardiovascular homeostasis, and CNS homeostasis as ACE-2 confer redox homeostasis by mitigating Ang-II-induced oxidative stress (146). However, in COVID-19, ACE-2 acts as receptor on human respiratory epithelial cells for SARS-CoV-2 binding (7). A recent report by Markus Hoffmann et al. (7)

provided evidence that the SARS-CoV-2 strain use its spike (S)-protein to bind to ACE-2. Authors of this paper have also demonstrated the efficiency of TMPRSS2 in SARS-CoV-2 viral strain priming in host cells (7). Therefore, targeting ACE-2 could be a viable strategy to prevent the entry of SARS-CoV-2 into the human system. However, a recent report by Guan et al. (147) cautioned that the administration of ACE inhibitors significantly induced adverse clinical outcomes in COVID-19 patients due to severe hypertension, coronary artery disease, and chronic renal failure; hence, further use of ACE inhibitors to treat COVID-19 infections was halted (147–149). In another report Diaz (149) hypothesized that COVID-19 patients receiving I.V. infusions of ACEIs and ARBs (AT1- Receptor Blockers) are at a higher risk of attaining severe disease pathogenesis. Hence, they supported the development of NSMIs such as "TMPRSS2 inhibitors to treat SARS-CoV-2 infections (7)".

Reasons for the Failure of Current Therapeutic Modalities Against SARS-CoV-2

The failure of disease management and lack of selective therapies could be due to the intricate COVID-19 pathogenesis induced by the SARS-CoV-2 infection. Hence, the early recognition of disease is essential for effective management of COVID-19 (48).

Although, several reports delineated the efficacy of certain NSMIs viz., *ribavirin, promazine*, and *IMP dehydrogenase inhibitors* to inhibit *in vivo* models of SARS- CoV replication, later, they were proven ineffective (60, 150–152). A report by Reghunathan et al. (153) showed that the immune response produced against SARS-CoV may be different from other viral infections as indicated by the lack of upregulation in MHC-I genes, cytokines, and IFNs or complement-mediated cytolysis in peripheral blood mononuclear cells (PBMCs). The failure in the development of a vaccine is due to antibody-dependent enhancement and Th-2 immunopathology (154–156).

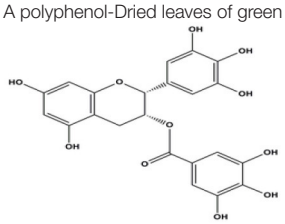
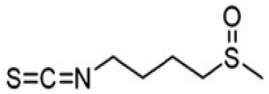
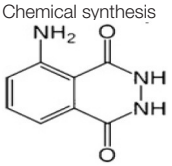
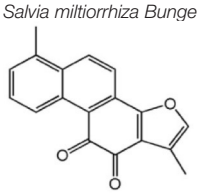
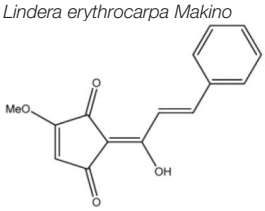
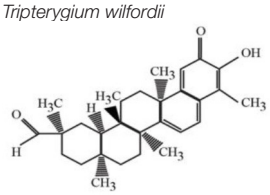
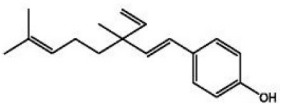
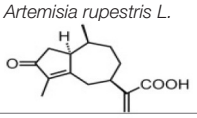
Pegylated IFN-α inhibits viral replication of SARS-CoV and offers protection against type I pneumocytes in lungs (2). A significant reason for the failure or lack of selective therapies against SARS-CoV-2-induced SARS is the intricate immune system mediated pathophysiology (4). Other reports by Law et al., also detailed similar mechanisms (157, 158). SARS-CoV can evade host IFN-mediated viral growth inhibition by activating IFN-regulatory factor 3 (157). Furthermore, SARS-CoV could induce apoptosis in lymphocytes *in vitro* using "*ORF 7a, ORF 3a, and ORF 3b, E protein, and N protein*" (159–162). For instance, the SARS-CoV can evade immunity as indicated by the decline in CD4 and CD8 T cells (163). Therefore, it is necessary to uncover the complement-based cytolysis in human patients in response to the SARS-CoV-2 strain as this virus executes unusual mechanisms to evade the human immune system consequently inducing pathogenesis and mortality. The prospective research should focus on this viral-mediated immune signaling with respect to SARS-CoV for developing effective NSMIs.

Intravenous (IV) hyperimmune globulin therapy is one of the immunotherapies known to downmodulate pro-inflammatory cytokines and mitigate the severity of infection in COVID-19 patients. IV infusion of immunoglobulins composed of a high dose of antibodies, which can bind to a number of inhibitory receptors viz., Fc gamma receptor IIB (FcγRIIB) (164, 165) and FcγRIIC (166) and confer anti-inflammatory responses against SARS-CoV-2 (Completed Clinical Trials: hyperimmune plasma NCT04321421).

Nrf-2 Modulators vs. SARS-CoV-2 Induced Oxidative Stress

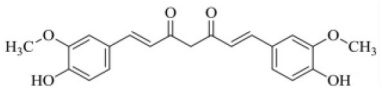
Oxidative stress is significantly induced by several viral infections inside the lungs through the downregulation of redox regulator nuclear factor-erythroid 2 related factor 2 (Nrf-2) (167). Nrf-2 is a leucine-zipper transcription factor (167) expressed predominantly in nasal epithelium, epithelial cells of lungs, and alveolar macrophages (168, 169). Disruption of Nrf-2 and Keap1 interaction triggers the activation of the anti-oxidant defense mechanism (169). For instance, Nrf-2 activation offers protection against inflammation and lung injury induced by influenza viral infections and respiratory syncytial virus (RSV) through the anti-oxidant defense pathway (170). Several viral proteins in the host cells can foster optimum levels of ROS-mediated oxidative stress to facilitate viral metabolism and the viral replication cycle without killing host cells (168, 171–174). Recent seminal studies described the active role of viruses in inhibiting the Nrf-2 pathway (175–177). For instance, the positive regulation of Nrf-2 in modulating the thiol redox system and oxidative stress for the survival of infected astrocytes was observed in *Moloney murine leukemia virus and HIV virus* (178). The HCV virus could induce the downregulation of Nrf-2 dependent NQO1, GCLC, and GPx and modulate oxidative stress (179, 180). An RSV infection mediates proteasomal degradation, deacetylation, and SUMOylation of Nrf-2 consequently causing the downregulation of NQO1, CAT, and SOD1 gene expression (181). Hence, Nrf-2 activators are potential anti-viral agents, which can be tested against the SARS-CoV-2 infection (167). Future research is highly imperative in unraveling the underlying activity of Nrf-2 for emerging SARS-CoV-2 survival by analyzing Nrf2 target genes NQO1, GCLC, and GPx. In addition, the SARS-CoV-2 mediated expression of serine and cysteine proteases in different cell lines should be investigated in relation to Nrf-2 activation, which is a beneficial strategy to combating SARS-CoV-2 pathogenesis. However, in the case of certain viral infections, it is imperative to develop Nrf-2 inhibitors to protect the host cells (182). For instance, the Marburg virus (a causative agent for lethal hemorrhagic fever) can modulate oxidative stress by activating Nrf-2 dependent signaling through the blockade of "VP-24 viral protein" binding to KEAP1 (183). Therefore, VP-24 dependent Nrf2 activation can mediate the upregulation of genes HO (heme oxygenase)-1, NQO1, and GCLM (183). In the case of Dengue virus, the viral particles could induce ER stress and activate Nrf-2 signaling, which then lead to TNF-α secretion (184). In this scenario, it is crucial to uncover any underlying mechanisms of emerging SARS-CoV-2 survival through the modulation of oxidative stress via Nrf-2 signaling in different cells of different organs including lungs (183). The prospective

TABLE 4 | Structure and mechanism of action of naturally occurring Nrf2 modulators.

Nrf-2 modulators effective against viruses	Mechanism of Action	Source and structure	References
EGCG	Inhibits viral replication of influenza A/Bangkok/1/79 infection in lung cells Inhibits Tat-induced HIV-1 infection	A polyphenol-Dried leaves of green tea 	(167) (185) (202)
SFN	Inhibits viral replication by enhancing expression of Nrf-2 expression, and antiviral mediators viz., RIG-1, IFN-β, and MxA.	Isothiocyanate—cruciferous vegetables 	(185) (203)
α-luminol (monosodium α-luminol)	Inhibits MoMuL virus	Chemical synthesis 	(191)
Tanshinone IIA	Inhibits Tat-induced HIV-1 via Nrf-2 upregulation	*Salvia miltiorrhiza Bunge* 	(204) (204)
Lucidone	Inhibits Dengue virus HCV (Hepatitis C Virus) growth	*Lindera erythrocarpa Makino* 	(195)
Celastrol (quinone methide triterpene)	Inhibits Tat-induced HIV-1 infection	*Tripterygium wilfordii* 	(197) (205)
Bakuchiol (phenolic isoprenoid)	Inhibits **influenza A H1N1** lung virus infection	*Psoralea corvlifolia L.* 	(199) (206)
Rupestonic acid (sesquiterpene)	Inhibits **influenza A (H1N1)** lung virus infection	*Artemisia rupestris L.* 	(207)

(Continued)

TABLE 4 | Continued

Nrf-2 modulators effective against viruses	Mechanism of Action	Source and structure	References
Curcumin	Inhibits **influenza A (H1N1)** lung virus infection	*Turmeric* 	(208) (201)

research studies should focus on the development of Nrf-2 modulators against SARS-CoV-2.

Natural Nrf-2 Modulators vs. Viral Infections

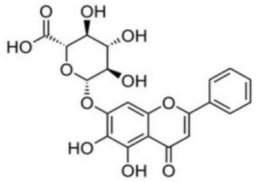
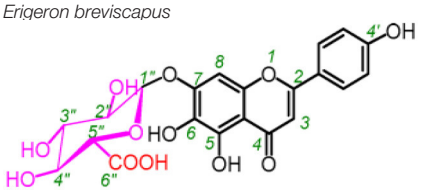
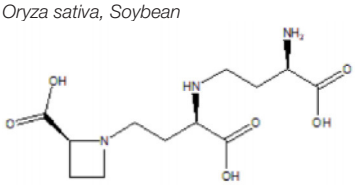
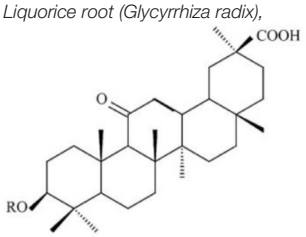
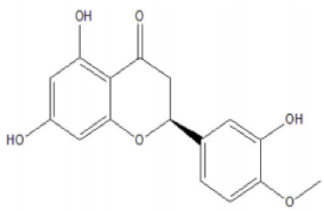
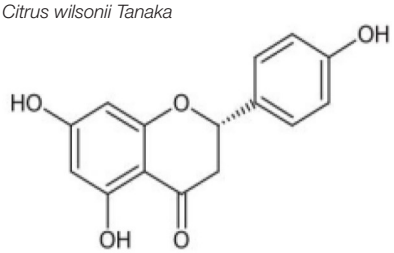
Natural products were proven to offer protection against virus-induced oxidative stress by modulating anti-oxidant defense pathways (185, 186). For instance, the administration of EGCG has mitigated viral replication of "influenza A/Bangkok/1/79 infection" by activating Nrf-2 to attenuate virus-induced oxidative stress, inflammation, and apoptosis in lung cells (186). Similarly, the cytoprotective and antioxidant efficacy of Nrf-2 was reported against PR8 influenza-A viral infection in AT-I and AT-II cells (186). Prospective research should focus on testing the efficacy of several natural products to block SARS-CoV-2 viral replication by ascertaining Nrf-2 mediated antioxidant responses. Studies have also shown the activation of host cellular transmembrane proteases (for example, serine proteases, cysteine proteases), which can further foster a prompt viral entry and viral replication in host cells by reducing Nrf-2 expression (4). Decline in proteolysis of the above proteases can actuate the propagation of several human viruses viz., Influenza, HIV, NIpah, Ebola, and Coronaviruses (SARS-CoV, MERS-CoV, SARS-CoV-2) (42, 90, 187–189). In this scenario, similar to influenza-A virus (190), it is highly important to unravel the influence of Nrf-2 expression on *TMPRSS2, and human airway trypsin-like protease* during SARS-CoV-2-mediated inflammatory conditions and oxidative stress in lungs. The downregulation of the Nrf-2 gene is correlated to serine protease activity and consequent influenza viral entry (185). Recent studies have demonstrated the efficacy of natural Nrf-2 activators viz., EGCG and sulforaphane (SFN) for blocking viral entry/viral replication as well as promoting antiviral mediators RIG-I, IFN-β, and MxA (185). In this context, it is essential to demonstrate the effects of nutritional interventions like SFN and EGCG against SARS-CoV-2 induced oxidative stress by modulating Nrf-2 signaling.

Evidence has demonstrated the use of naturally occurring Nrf2 activators for mitigating viral infections/post-viral infection induced complications. For example, α-luminol is a natural Nrf-2 activator, which confers the protection of astrocytes against the MoMuL virus (191). EGCG enhances nuclear Nrf-2 levels during Tat-induced HIV-1 infection and offers protection against virus induced oxidative stress (192). Tanshinone II A can induce upregulation of Nrf-2 expression and mitigates ROS production during Tat-induced HIV-1 infection via modulating *AMPK/Nampt/SIRT1* signaling in host cells (193). SFN enhances the phagocytic function of "HIV-infected alveolar macrophages in lungs" by activating Nrf-2 signaling, which further induces downstream antioxidant cascades (194). Lucidone is effective for the Nrf-2 mediated blockade of Dengue virus by inducing heme oxygenase-1 (195); rographolide could induce Nrf-2 induced antioxidant defenses against influenza A in lung cells (196); celastrol can mediate Nrf-2 induced antioxidant defenses against HIV-1 Tat-induced inflammation (197). Broccoli sprouts containing SFN acts as a Nrf-2 activator to reduce influenza-induced infection in lung cells (198). Bakuchiol and Rupestonic acid are phytoconstituents that confer Nrf-2 activation thereby promoting NQO1 gene expression and HO-1-mediated interferon activity to enhance antioxidant response against influenza virus in lung cells (199, 200). Curcumin is another significant compound that can modulate Nrf-2 signaling and enhance the generation of IFN-β to offer protection against the influenza virus (201). Curcumin can mitigate this viral infection by modulating *TLR2/4, p38/JNK MAPK, and NF-κB pathways* (201). However, studies are required to decipher the activity of these phytochemicals against SARS-CoV-2 (**Table 4**). A recent report by Drăgoi (209) hypothesized that the potent natural Nrf-2 activators viz., resveratrol, SFN, curcumin, and Asea redox should be evaluated in different combinations with conventional drugs against SARS-CoV-2 infection in both *in vitro* and *in vivo* models and to further deduce a correlation between Nrf-2 activity and SARS-CoV-2 viral entry/replication.

Naturally Occurring Small Molecule Inhibitors vs. SARS-CoV-2

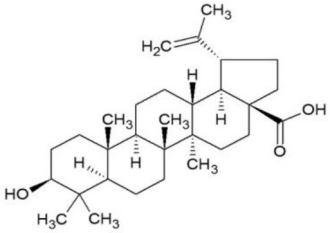
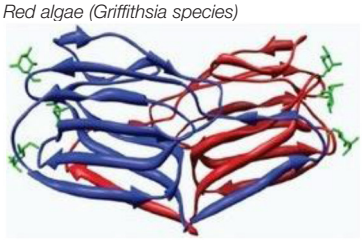
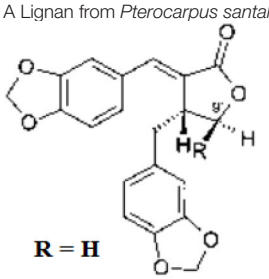
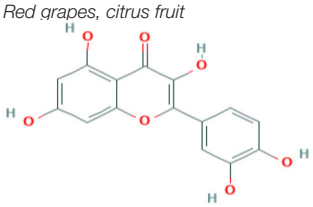
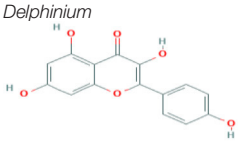
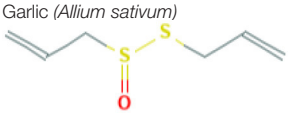
SARS-CoV-2 is progressively inducing a high mortality rate across the globe due to the lack of selective therapeutic interventions or vaccination. Recent reports by Lu et al. (210) and Xu et al. (148) delineated that the S-protein of SARS-CoV-2 and SARS Co-V exhibit similar 3-D pharmacophore in the receptor binding domain (RBD) of ACE-2 of human cells. COVID-19 patients are characterized by the severe viral pathogenesis due to extensive cytokine storm viz., TNF-α, IL-1β, IL-10, IFNγ, and MCP-1 in infected lung tissues (48). A report by Chen and Du (211) hypothesized that the phyto-constituents such as *"baicalin, scutellarin, hesperetin, nicotianamine, and glycyrrhizin*" may deliver anti-SARS-CoV-2 effects. Hesperetin glycoside abundant in citrus fruits, which can inhibit the SARS-CoV 3CLpro (212). The activity of this molecule must be examined against serine/cysteine proteases, which support SARS-CoV-2 viral entry/replication. Traditional citrus flavonoids were

TABLE 5 | Structure and mechanism of action of NSMIs identified against SARS-CoV2 using molecular docking studies.

NSMIs Identified using Molecular Docking	Mechanism of Action	Source and Structure	References
Baicalin (a flavonoid)	Predicted to exhibit a capacity for binding to ACE-2 for inducing anti-SARS-CoV-2 effects	*Scutellaria baicalensis* 	(211, 217)
Scutellarin (a flavone glycoside)	Predicted to exhibit a capacity for binding to ACE-2 to induce anti-SARS-CoV-2 effects	*Erigeron breviscapus* 	(211, 218)
Nicotianamine	Predicted to exhibit a capacity for binding to ACE-2 to induce anti-SARS-CoV-2 effects	Leaves of *L. chinense* *Fagus sylvatica, Avena sativa* *Oryza sativa, Soybean* 	(211, 219, 220)
Glycyrrhizin	Predicted to exhibit a capacity for binding to ACE-2 to induce anti-SARS-CoV-2 effects Note: Yet to be examined against SARS-CoV-2 infection	*Liquorice root (Glycyrrhiza radix),* 	(211)
Hesperetin glycoside	Potent inhibitor of SARS-CoV 3CLpro Note: Yet to be examined against SARS-CoV-2 infection	*Citrus aurantium* *Citri Reticulatae Pericarpium* 	(211, 212)
Naringenin	Binds to ACE-2, a receptor for SARS-CoV-2	*Citrus wilsonii Tanaka* 	(211, 213)

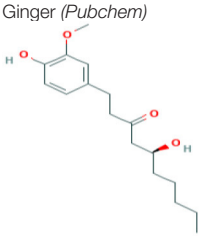
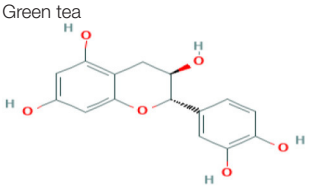
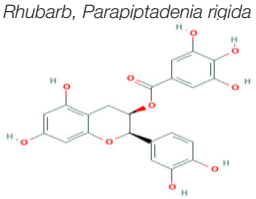
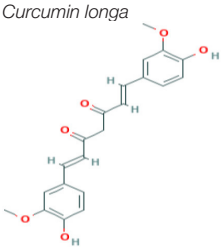
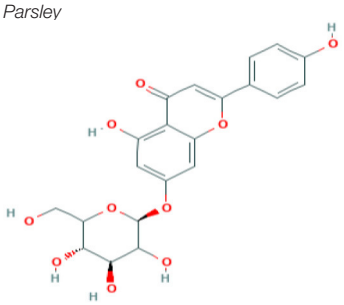
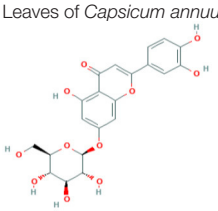
(Continued)

TABLE 5 | Continued

NSMIs Identified using Molecular Docking	Mechanism of Action	Source and Structure	References
Betulinic acid	Competitively inhibits SARS-CoV 3CL protease	*Outer bark of the birches (Betula)* 	(214, 221)
Griffithsin	Binds to the SARS-CoV spike (S) -protein and inhibit viral entry	*Red algae (Griffithsia species)* 	(215, 222)
Savinin	Competitively inhibits SARS-CoV 3CL protease	A Lignan from *Pterocarpus santalinus* 	(214)
Quercetin	Predicted to inhibit SARS-CoV-2 6LU7 Main protease (Mpro)	*Red grapes, citrus fruit*  *(Pubchem)*	(65)
Kaempferol	Predicted to inhibit SARS-CoV-2 6LU7 Main protease (Mpro)	*Delphinium*  *(Pubchem)*	(65)
Allicin	Predicted to inhibit SARS-CoV-2 6LU7 Main protease (Mpro)	Garlic *(Allium sativum)*  *(Pubchem)*	(65)

(Continued)

TABLE 5 | Continued

NSMIs Identified using Molecular Docking	Mechanism of Action	Source and Structure	References
Gingerol	Predicted to inhibit SARS-CoV-2 6LU7 Main protease (Mpro)	Ginger *(Pubchem)* 	(65)
Catechin	Predicted to inhibit SARS-CoV-2 6LU7 Main protease (Mpro)	Green tea  *(Pubchem)*	(65)
Epicatechingallate	Predicted to inhibit SARS-CoV-2 6LU7 Main protease (Mpro)	*Rhubarb, Parapiptadenia rigida*  *(Pubchem)*	(65)
Curcumin	Predicted to inhibit SARS-CoV-2 6LU7 Main protease (Mpro)	*Curcumin longa*  *(Pubchem)*	(65)
Apigenin-7- glucoside	Predicted to inhibit SARS-CoV-2 6LU7 Main protease (Mpro)	*Parsley*  *(Pubchem)*	(65)
Luteolin-7- glucoside	Predicted to inhibit SARS-CoV-2 6LU7 Main protease (Mpro)	Leaves of *Capsicum annuum*  *(Pubchem)*	(65)

reported to have a potential to act against SARS-CoV-2 as studied by molecular docking studies. Molecular docking simulations, LC-MS studies described the efficacy of citrus flavonoids (ex. naringenin) in binding to ACE-2, and mitigating inflammation-induced lung injury by the SARS-CoV-2 virus (213). Further studies should evaluate these compounds in preclinical models to determine the safety and efficacy against the SARS-CoV-2 infection.

Natural products such as di/tri-terpenoids, lignoids were proven to inhibit the viral replication of coronaviruses *in vitro* (214); griffithsin could block coronaviral entry by binding to the SARS-CoV spike glycoprotein (215). TSL-1 can block coronaviral entry/replication; Leaf extracts of *Toona sinesis Roem* effectively blocked SARS-CoV replication (57). Betulinic acid, savinin can act as competitive inhibitors against SARS-CoV 3CL protease to block viral entry (214). The research gap must be filled to develop nutritional therapeutic interventions by investigating the efficacy of these phytochemicals against SARS-CoV-2 viral entry. Seeds of *Psorelia corylifolia* exhibit inhibitory effects against the SARS-CoV papain-like protease required for coronavirus entry/replication. The efficacy of these molecules should be examined against SARS-CoV-2 (216) (**Table 5**). The active site pockets of main proteases such as 6LU7 and 2 GTB in SARS-CoV-2 are reported to be involved in conferring viral entry/fusion; hence, these sites should be considered as the potential drug targets against SARS-CoV-2 (66). A molecular docking study by Khaerunnisa et al. (65) reported the predicted efficacy of bioactive compounds against above SARS-CoV-2 main protease (Mpro) sites viz., "*nelfinavir, lopinavir, kaempferol, quercetin, luteolin-7-glucoside, demethoxycurcumin, naringenin, apigenin-7-glucoside, oleuropein, curcumin, catechin, epicatechin-gallate, zingerol, gingerol, and allicin*" (**Table 5**).

CONCLUSIONS

The life-threatening consequences of the COVID-19 pandemic remain high due to lack of selective targeted therapies and vaccination strategies. This is primarily due to extreme genomic variability of RNA viruses as well as variations in the host-cell invading mechanisms. Hence, this review benefits virologists, medical scientists, and cell biologists to ascertain and develop NSMIs, Nrf-2 modulators, and clinically viable vaccines to combat this devastating SARS-CoV-2 strain. However, many more preclinical and clinical studies are required to uncover the therapeutic efficacy of potential phytochemicals, natural Nrf2 modulators, and several NSMIs against the SARS-CoV-2 infection. Furthermore, studies are also warranted to overcome ADE responses, and Th-2 immunopathology for the development of safe and efficacious vaccines against SARS-CoV-2. In summary, this review provides an overview on the existing knowledge and shows directions to various areas that require immediate attention.

AUTHOR CONTRIBUTIONS

NB, SS, and SM: idea development, data collection, manuscript preparation, writing, and proof reading. AS, VN, and LM: cross referencing, data collection, and proof reading. SM, GA, and RR: editing, literature search, and proof reading. SS: execution of the literature search. All authors contributed to the article and approved the submitted version.

REFERENCES

1. Guan Y, Zheng B, He Y, Liu X, Zhuang Z, Cheung C, et al. Isolation and characterization of viruses related to the SARS coronavirus from animals in southern China. *Science.* (2003) 302:276–8. doi: 10.1126/science.1087139
2. Haagmans BL, Kuiken T, Martina BE, Fouchier RA, Rimmelzwaan GF, Van Amerongen G, et al. Pegylated interferon-α protects type 1 pneumocytes against SARS coronavirus infection in macaques. *Nat Med.* (2004) 10:290–3. doi: 10.1038/nm1001
3. Weiss SR, Navas-Martin S. Coronavirus pathogenesis and the emerging pathogen severe acute respiratory syndrome coronavirus. *Microbiol Mol Biol Rev.* (2005) 69:635–64. doi: 10.1128/MMBR.69.4.635-664.2005
4. Cascella M, Rajnik M, Cuomo A, Dulebohn SC, Di Napoli R. *Features, Evaluation and Treatment Coronavirus (COVID-19)*. StatPearls Publishing (2020).
5. Chan JF-W, Kok K-H, Zhu Z, Chu H, To K-W, Yuan S, et al. Genomic characterization of the 2019 novel human-pathogenic coronavirus isolated from a patient with atypical pneumonia after visiting Wuhan. *Emerg Microbes Infect.* (2020) 9:221–36. doi: 10.1080/22221751.2020.1719902
6. Li Q, Guan X, Wu P, Wang X, Zhou L, Tong Y, et al. Early transmission dynamics in Wuhan, China, of novel coronavirus–infected pneumonia. *Ne Engl J Med.* (2020) 382:1199–207. doi: 10.1056/NEJMoa2001316
7. Hoffmann M, Kleine-Weber H, Krüger N, Mueller MA, Drosten C, Pöhlmann S. The novel coronavirus 2019 (2019-nCoV) uses the SARS-coronavirus receptor ACE2 and the cellular protease TMPRSS2 for entry into target cells. *BioRxiv*. (2020). doi: 10.1101/2020.01.31.929042
8. Corman V, Lienau J, Witzenrath M. Coronaviruses as the cause of respiratory infections. *Der Internist*. (2019) 60:1136–45. doi: 10.1007/s00108-019-00671-5
9. Fehr AR, Channappanavar R, Perlman S. Middle East respiratory syndrome: emergence of a pathogenic human coronavirus. *Ann Rev Med.* (2017) 68:387–99. doi: 10.1146/annurev-med-051215-031152
10. Organization WH. *Laboratory Testing for Coronavirus Disease 2019 (COVID-19) in Suspected Human Cases: Interim Guidance*. World Health Organization (2020).
11. de Wit E, van Doremalen N, Falzarano D, Munster VJ. SARS and MERS: recent insights into emerging coronaviruses. *Nat Rev Microbiol.* (2016) 14:523. doi: 10.1038/nrmicro.2016.81
12. Wang C, Horby PW, Hayden FG, Gao GF. A novel coronavirus outbreak of global health concern. *Lancet.* (2020) 395:470–3. doi: 10.1016/S0140-6736(20)30185-9
13. Tian S, Hu W, Niu L, Liu H, Xu H, Xiao S-Y. Pulmonary pathology of early phase 2019 novel coronavirus (COVID-19) pneumonia in two patients with lung cancer. *J Thorac Oncol*. (2020). doi: 10.20944/preprints202002.0220.v2
14. Menachery VD, Yount BL, Josset L, Gralinski LE, Scobey T, Agnihothram S, et al. Attenuation and restoration of severe acute respiratory syndrome coronavirus mutant lacking 2′-O-methyltransferase activity. *J Virol.* (2014) 88:4251–64. doi: 10.1128/JVI.03571-13
15. Miura TA, Wang J, Holmes KV, Mason RJ. Rat coronaviruses infect rat alveolar type I epithelial cells and induce expression of CXC chemokines. *Virol.* (2007) 369:288–98. doi: 10.1016/j.virol.2007.07.030
16. Abu-Harb M, Bell F, Finn A, Rao W, Nixon L, Shale D, et al. IL-8 and neutrophil elastase levels in the respiratory tract of infants with RSV bronchiolitis. *Eur Respir J.* (1999) 14:139–43. doi: 10.1034/j.1399-3003.1999.14a23.x
17. Tumpey TM, García-Sastre A, Taubenberger JK, Palese, Swayne DE, Pantin-Jackwood MJ, et al. Pathogenicity of influenza viruses with genes from the 1918 pandemic virus: functional roles of alveolar macrophages and

neutrophils in limiting virus replication and mortality in mice. *J Virol.* (2005) 79:14933–44. doi: 10.1128/JVI.79.23.14933-14944.2005
18. Turner RB. The role of neutrophils in the pathogenesis of rhinovirus infections. *Pediatr Infect Dis J.* (1990) 9:832–5.
19. Wang S, Xu H, Wraith A, Bowden J, Alpers J, Forsyth K. Neutrophils induce damage to respiratory epithelial cells infected with respiratory syncytial virus. *Eur Respir J.* (1998) 12:612–8. doi: 10.1183/09031936.98.12030612
20. Zhang Y, Luxon BA, Casola A, Garofalo RP, Jamaluddin M, Brasier AR. Expression of respiratory syncytial virus-induced chemokine gene networks in lower airway epithelial cells revealed by cDNA microarrays. *J Virol.* (2001) 75:9044–58. doi: 10.1128/JVI.75.19.9044-9058.2001
21. Zhu Z, Tang W, Gwaltney JM Jr., Wu Y, Elias JA. Rhinovirus stimulation of interleukin-8 *in vivo* and *in vitro*: role of NF-κB. *Am J Physiol Lung Cell Mol Physiol.* (1997) 273:L814–24. doi: 10.1152/ajplung.1997.273.4.L814
22. Stievano L, Piovan E, Amadori A. C and CX 3 C chemokines: cell sources and physiopathological implications. *Crit Rev Immunol.* (2004) 24:205–28. doi: 10.1615/CritRevImmunol.v24.i3.40
23. Vanderbilt JN, Mager EM, Allen L, Sawa T, Wiener-Kronish J, Gonzalez R, et al. CXC chemokines and their receptors are expressed in type II cells and upregulated following lung injury. *Am J Respir Cell Mol Biol.* (2003) 29:661–8. doi: 10.1165/rcmb.2002-0227OC
24. Londhe VA, Belperio JA, Keane MP, Burdick MD, Xue YY, Strieter RM. CXCR2 is critical for dsRNA-induced lung injury: relevance to viral lung infection. *J Inflamm.* (2005) 2:4. doi: 10.1186/1476-9255-2-4
25. Yen Y-T, Liao F, Hsiao C-H, Kao C-L, Chen Y-C, Wu-Hsieh BA. Modeling the early events of severe acute respiratory syndrome coronavirus infection *in vitro*. *J Virol.* (2006) 80:2684–93. doi: 10.1128/JVI.80.6.2684-2693.2006
26. South AM, Diz D, Chappell MC. *COVID-19, ACE2 and the Cardiovascular Consequences*. Rockville, MD: American Physiological Society (2020).
27. Li W, Moore MJ, Vasilieva N, Sui J, Wong SK, Berne MA, et al. Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. *Nature.* (2003) 426:450–4. doi: 10.1038/nature02145
28. Kuba K, Imai Y, Rao S, Gao H, Guo F, Guan B, et al. A crucial role of angiotensin converting enzyme 2 (ACE2) in SARS coronavirus–induced lung injury. *Nat Med.* (2005) 11:875–9. doi: 10.1038/nm1267
29. Hofmann H, Pyrc K, van der Hoek L, Geier M, Berkhout B, Pöhlmann S. Human coronavirus NL63 employs the severe acute respiratory syndrome coronavirus receptor for cellular entry. *Proc Natl Acad Sci USA.* (2005) 102:7988–93. doi: 10.1073/pnas.0409465102
30. Yeager CL, Ashmun RA, Williams RK, Cardellichio CB, Shapiro LH, Look AT, et al. Human aminopeptidase N is a receptor for human coronavirus 229E. *Nature.* (1992) 357:420–2. doi: 10.1038/357420a0
31. van der Hoek L, Pyrc K, Jebbink MF, Vermeulen-Oost W, Berkhout RJ, Wolthers KC, et al. Identification of a new human coronavirus. *Nat Med.* (2004) 10:368–73. doi: 10.1038/nm1024
32. Kolb AF, Hegyi A, Siddell SG. Identification of residues critical for the human coronavirus 229E receptor function of human aminopeptidase N. *J Gen Virol.* (1997) 78:2795–802. doi: 10.1099/0022-1317-78-11-2795
33. Michel N, Allespach I, Venzke S, Fackler OT, Keppler OT. The Nef protein of human immunodeficiency virus establishes superinfection immunity by a dual strategy to downregulate cell-surface CCR5 and CD4. *Curr Biol.* (2005) 15:714–23. doi: 10.1016/j.cub.2005.02.058
34. Wevers BA, van der Hoek L. Renin–angiotensin system in human coronavirus pathogenesis. *Fut Virol.* (2010) 5:145–161. doi: 10.2217/fvl.10.4
35. Chang Y-J, Liu CY-Y, Chiang B-L, Chao Y-C, Chen C-C. Induction of IL-8 release in lung cells via activator protein-1 by recombinant baculovirus displaying severe acute respiratory syndrome-coronavirus spike proteins: identification of two functional regions. *J Immunol.* (2004) 173:7602–14. doi: 10.4049/jimmunol.173.12.7602
36. Tripet B, Howard MW, Jobling M, Holmes RK, Holmes KV, Hodges RS. Structural characterization of the SARS-coronavirus spike S fusion protein core. *J Biol Chem.* (2004) 279:20836–49. doi: 10.1074/jbc.M400759200
37. Pyrc K, Dijkman R, Deng L, Jebbink MF, Ross HA, Berkhout B, et al. Mosaic structure of human coronavirus NL63, one thousand years of evolution. *J Mol Biol.* (2006) 364:964–73. doi: 10.1016/j.jmb.2006.09.074
38. Li W, Sui J, Huang I-C, Kuhn JH, Radoshitzky SR, Marasco WA, et al. The S proteins of human coronavirus NL63 and severe acute respiratory syndrome coronavirus bind overlapping regions of ACE2. *Virology.* (2007) 367:367–74. doi: 10.1016/j.virol.2007.04.035
39. Wu K, Li W, Peng G, Li F. Crystal structure of NL63 respiratory coronavirus receptor-binding domain complexed with its human receptor. *Proc Natl Acad Sci USA.* (2009) 106:19970–74. doi: 10.1073/pnas.0908837106
40. Haga S, Yamamoto N, Nakai-Murakami C, Osawa Y, Tokunaga K, Sata T, et al. Modulation of TNF-α-converting enzyme by the spike protein of SARS-CoV and ACE2 induces TNF-α production and facilitates viral entry. *Proc Natl Acad Sci USA.* (2008) 105:7809–14. doi: 10.1073/pnas.0711241105
41. Glowacka I, Bertram S, Herzog P, Pfefferle S, Steffen I, Muench MO, et al. Differential downregulation of ACE2 by the spike proteins of severe acute respiratory syndrome coronavirus and human coronavirus NL63. *J Virol.* (2010) 84:1198–205. doi: 10.1128/JVI.01248-09
42. Matsuyama S, Nagata N, Shirato K, Kawase M, Takeda M, Taguchi F. Efficient activation of the severe acute respiratory syndrome coronavirus spike protein by the transmembrane protease TMPRSS2. *J Virol.* (2010) 84:12658–64. doi: 10.1128/JVI.01542-10
43. Shirato K, Kawase M, Matsuyama S. Middle East respiratory syndrome coronavirus infection mediated by the transmembrane serine protease TMPRSS2. *J Virol.* (2013) 87:12552–61. doi: 10.1128/JVI.01890-13
44. Bertram S, Dijkman R, Habjan M, Heurich A, Gierer S, Glowacka I, et al. TMPRSS2 activates the human coronavirus 229E for cathepsin-independent host cell entry and is expressed in viral target cells in the respiratory epithelium. *J Virol.* (2013) 87:6150–60. doi: 10.1128/JVI.03372-12
45. Gierer S, Bertram S, Kaup F, Wrensch F, Heurich A, Krämer-Kühl A, et al. The spike protein of the emerging betacoronavirus EMC uses a novel coronavirus receptor for entry, can be activated by TMPRSS2, and is targeted by neutralizing antibodies. *J Virol.* (2013) 87:5502–11. doi: 10.1128/JVI.00128-13
46. Iwata-Yoshikawa N, Okamura T, Shimizu Y, Hasegawa H, Takeda M, Nagata N. TMPRSS2 contributes to virus spread and immunopathology in the airways of murine models after coronavirus infection. *J Virol.* (2019) 93:e01815–18. doi: 10.1128/JVI.01815-18
47. Ko W-C, Rolain J-M, Lee N-Y, Chen P-L, Huang C-T, Lee P-I, et al. Arguments in favor of remdesivir for treating SARS-CoV-2 infections. *Int J Antimicrob Agents.* (2020) 55:105933. doi: 10.1016/j.ijantimicag.2020.105933
48. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet.* (2020) 395:497–506. doi: 10.1016/S0140-6736(20)30183-5
49. Adedeji AO, Severson W, Jonsson C, Singh K, Weiss SR, Sarafianos SG. Novel inhibitors of severe acute respiratory syndrome coronavirus entry that act by three distinct mechanisms. *J Virol.* (2013) 87:8017–28. doi: 10.1128/JVI.00998-13
50. Åkerström S, Mirazimi A, Tan Y-J. Inhibition of SARS-CoV replication cycle by small interference RNAs silencing specific SARS proteins, 7a/7b, 3a/3b and S. *Antiviral Res.* (2007) 73:219–27. doi: 10.1016/j.antiviral.2006.10.008
51. Ratia K, Pegan S, Takayama J, Sleeman K, Coughlin M, Baliji S, et al. A noncovalent class of papain-like protease/deubiquitinase inhibitors blocks SARS virus replication. *Proc Natl Acad Sci USA.* (2008) 105:16119–24. doi: 10.1073/pnas.0805240105
52. Ghosh AK, Takayama J, Rao KV, Ratia K, Chaudhuri R, Mulhearn DC, et al. Severe acute respiratory syndrome coronavirus papain-like novel protease inhibitors: design, synthesis, protein– ligand X-ray structure and biological evaluation. *J Med Chem.* (2010) 53:4968–79. doi: 10.1021/jm1004489
53. Ghosh AK, Gong G, Grum-Tokars V, Mulhearn DC, Baker SC, Coughlin M, et al. Design, synthesis and antiviral efficacy of a series of potent chloropyridyl ester-derived SARS-CoV 3CLpro inhibitors. *Bioorg Med Chem Lett.* (2008) 18:5684–8. doi: 10.1016/j.bmcl.2008.08.082
54. Rogers J, Schoepp R, Schröder O, Clements T, Holland T, Li J, et al. Rapid discovery and optimization of therapeutic antibodies against emerging infectious diseases. *Protein Eng Design Select.* (2008) 21:495–505. doi: 10.1093/protein/gzn027
55. Stadler K, Ha HR, Ciminale V, Spirli C, Saletti G, Schiavon M, et al. Amiodarone alters late endosomes and inhibits SARS coronavirus infection at a post-endosomal level. *Am J Respir Cell Mol Biol.* (2008) 39:142–9. doi: 10.1165/rcmb.2007-0217OC
56. Khamitov R, Loginova S, Shchukina V, Borisevich S, Maksimov V, Shuster A. Antiviral activity of arbidol and its derivatives against the pathogen

of severe acute respiratory syndrome in the cell cultures. *Voprosy Virusol.* (2008) 53:9–13.
57. Chen C-J, Michaelis M, Hsu H-K, Tsai C-C, Yang KD, Wu Y-C, et al. Toona sinensis Roem tender leaf extract inhibits SARS coronavirus replication. *J Ethnopharmacol.* (2008) 120:108–11. doi: 10.1016/j.jep.2008.07.048
58. Gong J, Ju A, Zhou D, Li D, Zhou W, Geng W, et al. Salvianolic acid Y: a new protector of PC12 cells against hydrogen peroxide-induced injury from Salvia officinalis. *Molecules.* (2015) 20:683–92. doi: 10.3390/molecules20010683
59. Haga S, Nagata N, Okamura T, Yamamoto N, Sata T, Yamamoto N, et al. TACE antagonists blocking ACE2 shedding caused by the spike protein of SARS-CoV are candidate antiviral compounds. *Antiviral Res.* (2010) 85:551–5. doi: 10.1016/j.antiviral.2009.12.001
60. Barnard DL, Day CW, Bailey K, Heiner M, Montgomery R, Lauridsen L, et al. Evaluation of immunomodulators, interferons and known *in vitro* SARS-coV inhibitors for inhibition of SARS-coV replication in BALB/c mice. *Antiviral Chem Chemother.* (2006) 17:275–84. doi: 10.1177/095632020601700505
61. Kumaki Y, Day CW, Bailey KW, Wandersee MK, Wong M-H, Madsen JR, et al. Induction of interferon-γ-inducible protein 10 by SARS-CoV infection, interferon alfacon 1 and interferon inducer in human bronchial epithelial Calu-3 cells and BALB/c mice. *Antiviral Chem Chemother.* (2010) 20:169–77. doi: 10.3851/IMP1477
62. Scagnolari C, Vicenzi E, Bellomi F, Stillitano MG, Pinna D, Poli G, et al. Increased sensitivity of SARS-coronavirus to a combination of human type I and type II interferons. *Antivir Ther.* (2004) 9:1003–11.
63. Sainz B Jr., Mossel EC, Peters C, Garry RF. Interferon-beta and interferon-gamma synergistically inhibit the replication of severe acute respiratory syndrome-associated coronavirus (SARS-CoV). *Virology.* (2004) 329:11–7. doi: 10.1016/j.virol.2004.08.011
64. Wang M, Cao R, Zhang L, Yang X, Liu J, Xu M, et al. Remdesivir and chloroquine effectively inhibit the recently emerged novel coronavirus (2019-nCoV) *in vitro*. *Cell Res.* (2020) 30:269–71. doi: 10.1038/s41422-020-0282-0
65. Khaerunnisa S, Kurniawan H, Awaluddin R, Suhartati S, Soetjipto S. Potential inhibitor of COVID-19 main protease (Mpro) from several medicinal plant compounds by molecular docking study. *Europe PMC.* (2020) 1:1–4. doi: 10.20944/preprints202003.0226.v1
66. Xu Z, Peng C, Shi Y, Zhu Z, Mu K, Wang X, et al. Nelfinavir was predicted to be a potential inhibitor of 2019-nCov main protease by an integrative approach combining homology modelling, molecular docking and binding free energy calculation. *BioRxiv*. (2020). doi: 10.1101/2020.01.27.921627. Available online at: https://europepmc.org/article/ppr/ppr110446
67. Adedeji AO, Sarafianos SG. Antiviral drugs specific for coronaviruses in preclinical development. *Curr Opin Virol.* (2014) 8:45–53. doi: 10.1016/j.coviro.2014.06.002
68. Wong SK, Li W, Moore MJ, Choe H, Farzan M. A 193-amino acid fragment of the SARS coronavirus S protein efficiently binds angiotensin-converting enzyme 2. *J Biol Chem.* (2004) 279:3197–201. doi: 10.1074/jbc.C300520200
69. Richardson P, Griffin I, Tucker C, Smith D, Oechsle O, Phelan A, et al. Baricitinib as potential treatment for 2019-nCoV acute respiratory disease. *Lancet.* (2020) 395:e30–31. doi: 10.1016/S0140-6736(20)30304-4
70. Kono M, Tatsumi K, Imai AM, Saito K, Kuriyama T, Shirasawa H. Inhibition of human coronavirus 229E infection in human epithelial lung cells (L132) by chloroquine: involvement of p38 MAPK and ERK. *Antiviral Res.* (2008) 77:150–2. doi: 10.1016/j.antiviral.2007.10.011
71. Colson P, Rolain J-M, Lagier J-C, Brouqui P, Raoult D. Chloroquine and hydroxychloroquine as available weapons to fight COVID-19. *Int J Antimicrob Agents.* (2020) 55:105932. doi: 10.1016/j.ijantimicag.2020.105932
72. Kindrachuk J, Ork B, Hart BJ, Mazur S, Holbrook MR, Frieman MB, et al. Antiviral potential of ERK/MAPK and PI3K/AKT/mTOR signaling modulation for Middle East respiratory syndrome coronavirus infection as identified by temporal kinome analysis. *Antimicrobial Agents Chemother.* (2015) 59:1088–99. doi: 10.1128/AAC.03659-14
73. Schor S, Einav S. Repurposing of kinase inhibitors as broad-spectrum antiviral drugs. *DNA Cell Biol.* (2018) 37:63–9. doi: 10.1089/dna.2017.4033
74. Dyall J, Coleman CM, Hart BJ, Venkataraman T, Holbrook MR, Kindrachuk J, et al. Repurposing of clinically developed drugs for treatment of Middle East respiratory syndrome coronavirus infection. *Antimicrob Agents Chemother.* (2014) 58:4885–93. doi: 10.1128/AAC.03036-14
75. Dinarello CA. Biologic basis for interleukin-1 in disease. *Blood.* (1996) 87:2095–147. doi: 10.1182/blood.V87.6.2095.bloodjournal8762095
76. Dinarello CA. Differences between anti-tumor necrosis factor-alpha monoclonal antibodies and soluble TNF receptors in host defense impairment. *J Rheumatol Suppl.* (2005) 74:40–47.
77. Warren TK, Wells J, Panchal RG, Stuthman KS, Garza NL, Van Tongeren SA, et al. Protection against filovirus diseases by a novel broad-spectrum nucleoside analogue BCX4430. *Nature.* (2014) 508:402–5. doi: 10.1038/nature13027
78. Hiv LLSF. Designing defenses against deadly viruses. *Cell.* (2014) 157:279–81. doi: 10.1016/j.cell.2014.03.033
79. Taylor R, Kotian P, Warren T, Panchal R, Bavari S, Julander J, et al. BCX4430–a broad-spectrum antiviral adenosine nucleoside analog under development for the treatment of Ebola virus disease. *J Infect Public Health.* (2016) 9:220–6. doi: 10.1016/j.jiph.2016.04.002
80. Kido H, Okumura Y, Takahashi E, Pan H-Y, Wang S, Chida J, et al. Host envelope glycoprotein processing proteases are indispensable for entry into human cells by seasonal and highly pathogenic avian influenza viruses. *J Mol Genet Med.* (2009) 3:167. doi: 10.4172/1747-0862.1000029
81. Yamaya M, Shimotai Y, Hatachi Y, Kalonji NL, Tando Y, Kitajima Y, et al. The serine protease inhibitor camostat inhibits influenza virus replication and cytokine production in primary cultures of human tracheal epithelial cells. *Pulmon Pharmacol Ther.* (2015) 33:66–74. doi: 10.1016/j.pupt.2015.07.001
82. Hatesuer B, Bertram S, Mehnert N, Bahgat MM, Nelson PS, Pöhlman S, et al. Tmprss2 is essential for influenza H1N1 virus pathogenesis in mice. *PLoS Pathog.* (2013) 9:e1003774. doi: 10.1371/journal.ppat.1003774
83. Tarnow C, Engels G, Arendt A, Schwalm F, Sediri H, Preuss A, et al. TMPRSS2 is a host factor that is essential for pneumotropism and pathogenicity of H7N9 influenza A virus in mice. *J Virol.* (2014) 88:4744–51. doi: 10.1128/JVI.03799-13
84. Sakai K, Ami Y, Tahara M, Kubota T, Anraku M, Abe M, et al. The host protease TMPRSS2 plays a major role in *in vivo* replication of emerging H7N9 and seasonal influenza viruses. *J Virol.* (2014) 88:5608–16. doi: 10.1128/JVI.03677-13
85. Sakai K, Sekizuka T, Ami Y, Nakajima N, Kitazawa M, Sato Y, et al. A mutant H3N2 influenza virus uses an alternative activation mechanism in TMPRSS2 knockout mice by loss of an oligosaccharide in the hemagglutinin stalk region. *J Virol.* (2015) 89:5154–8. doi: 10.1128/JVI.00124-15
86. Bertram S, Heurich A, Lavender H, Gierer S, Danisch S, Perin P, et al. Influenza and SARS-coronavirus activating proteases TMPRSS2 and HAT are expressed at multiple sites in human respiratory and gastrointestinal tracts. *PLoS ONE.* (2012) 7:e35876. doi: 10.1371/journal.pone.0035876
87. Glowacka I, Bertram S, Müller MA, Allen P, Soilleux E, Pfefferle S, et al. Evidence that TMPRSS2 activates the severe acute respiratory syndrome coronavirus spike protein for membrane fusion and reduces viral control by the humoral immune response. *J Virol.* (2011) 85:4122–34. doi: 10.1128/JVI.02232-10
88. Heurich A, Hofmann-Winkler H, Gierer S, Liepold T, Jahn O, Pöhlmann S. TMPRSS2 and ADAM17 cleave ACE2 differentially and only proteolysis by TMPRSS2 augments entry driven by the severe acute respiratory syndrome coronavirus spike protein. *J Virol.* (2014) 88:1293–307. doi: 10.1128/JVI.02202-13
89. Shirato K, Kawase M, Matsuyama S. Wild-type human coronaviruses prefer cell-surface TMPRSS2 to endosomal cathepsins for cell entry. *Virology.* (2018) 517:9–15. doi: 10.1016/j.virol.2017.11.012
90. Shulla A, Heald-Sargent T, Subramanya G, Zhao J, Perlman S, Gallagher T. A transmembrane serine protease is linked to the severe acute respiratory syndrome coronavirus receptor and activates virus entry. *J Virol.* (2011) 85:873–82. doi: 10.1128/JVI.02062-10
91. Bertram S, Glowacka I, Müller MA, Lavender H, Gnirss K, Nehlmeier I, et al. Cleavage and activation of the severe acute respiratory syndrome coronavirus spike protein by human airway trypsin-like protease. *J Virol.* (2011) 85:13363–72. doi: 10.1128/JVI.05300-11
92. Simmons G, Zmora P, Gierer S, Heurich A, Pöhlmann S. Proteolytic activation of the SARS-coronavirus spike protein: cutting enzymes at

the cutting edge of antiviral research. *Antiviral Res.* (2013) 100:605–14. doi: 10.1016/j.antiviral.2013.09.028
93. Lappi-Blanco E, Soini Y, Kinnula V, Pääkkö P. VEGF and bFGF are highly expressed in intraluminal fibromyxoid lesions in bronchiolitis obliterans organizing pneumonia. *J Pathol.* (2002) 196:220–7. doi: 10.1002/path.1038
94. van den Brand JM, Smits SL, Haagmans BL. Pathogenesis of Middle East respiratory syndrome coronavirus. *J Pathol.* (2015) 235:175–84. doi: 10.1002/path.4458
95. Chen F, Chan K, Jiang Y, Kao R, Lu H, Fan K, et al. *In vitro* susceptibility of 10 clinical isolates of SARS coronavirus to selected antiviral compounds. *J Clin Virol.* (2004) 31:69–75. doi: 10.1016/j.jcv.2004.03.003
96. Cinatl J, Morgenstern B, Bauer G, Chandra P, Rabenau H, Doerr H. Treatment of SARS with human interferons. *Lancet.* (2003) 362:293–4. doi: 10.1016/S0140-6736(03)13973-6
97. Ströher U, DiCaro A, Li Y, Strong JE, Aoki F, Plummer F, et al. Severe acute respiratory syndrome-related coronavirus is inhibited by interferon-α. *J Infect Dis.* (2004) 189:1164–7. doi: 10.1086/382597
98. Zheng B, He M-L, Wong K-L, Lum CT, Poon LL, Peng Y, et al. Potent inhibition of SARS-associated coronavirus (SCOV) infection and replication by type I interferons (IFN-α/β) but not by type II interferon (IFN-γ). *J Interferon Cytok Res.* (2004) 24:388–90. doi: 10.1089/1079990041535610
99. Satija N, Lal SK. The molecular biology of SARS coronavirus. *Ann N Y Acad Sci.* (2007) 1102:26–38. doi: 10.1196/annals.1408.002
100. Chen S, Luo H, Chen L, Chen J, Shen J, Zhu W, et al. An overall picture of SARS coronavirus (SARS-CoV) genome-encoded major proteins: structures, functions and drug development. *Curr Pharma Design.* (2006) 12:4539–53. doi: 10.2174/138161206779010459
101. Zhao G, Shi S-Q, Yang Y, Peng J-P. M and N proteins of SARS coronavirus induce apoptosis in HPF cells. *Cell Biol Toxicol.* (2006) 22:313–22. doi: 10.1007/s10565-006-0077-1
102. Chan C-M, Ma C-W, Chan W-Y, Chan HYE. The SARS-Coronavirus Membrane protein induces apoptosis through modulating the Akt survival pathway. *Arch Biochem Biophys.* (2007) 459:197–207. doi: 10.1016/j.abb.2007.01.012
103. Saikatendu KS, Joseph JS, Subramanian V, Neuman BW, Buchmeier MJ, Stevens RC, et al. Ribonucleocapsid formation of severe acute respiratory syndrome coronavirus through molecular action of the N-terminal domain of N protein. *J Virol.* (2007) 81:3913–21. doi: 10.1128/JVI.02236-06
104. Chen C-Y, Chang C-k, Chang Y-W, Sue S-C, Bai H-I, Riang L, et al. Structure of the SARS coronavirus nucleocapsid protein RNA-binding dimerization domain suggests a mechanism for helical packaging of viral RNA. *J Mol Biol.* (2007) 368:1075–86. doi: 10.1016/j.jmb.2007.02.069
105. Huang C, Ito N, C.-Tseng TK, Makino S. Severe acute respiratory syndrome coronavirus 7a accessory protein is a viral structural protein. *J Virol.* (2006) 80:7287–94. doi: 10.1128/JVI.00414-06
106. Yuan X, Wu J, Shan Y, Yao Z, Dong B, Chen B, et al. SARS coronavirus 7a protein blocks cell cycle progression at G0/G1 phase via the cyclin D3/pRb pathway. *Virology.* (2006) 346:74–85. doi: 10.1016/j.virol.2005.10.015
107. Vasilenko N, Moshynskyy I, Zakhartchouk A. SARS coronavirus protein 7a interacts with human Ap 4 A-hydrolase. *Virol J.* (2010) 7:31. doi: 10.1186/1743-422X-7-31
108. Li B-j, Tang Q, Cheng D, Qin C, Xie FY, Wei Q, et al. Using siRNA in prophylactic and therapeutic regimens against SARS coronavirus in Rhesus macaque. *Nat Med.* (2005) 11:944–51. doi: 10.1038/nm1280
109. Li T, Zhang Y, Fu L, Yu C, Li X, Li Y, et al. siRNA targeting the leader sequence of SARS-CoV inhibits virus replication. *Gene Ther.* (2005) 12:751–61. doi: 10.1038/sj.gt.3302479
110. Yi S, De Hua Y, Xiong J, Jie J, Huang B, Jin YX. Inhibition of genes expression of SARS coronavirus by synthetic small interfering RNAs. *Cell Res.* (2005) 15:193–200. doi: 10.1038/sj.cr.7290286
111. Wang Z, Ren L, Zhao X, Hung T, Meng A, Wang J, et al. Inhibition of severe acute respiratory syndrome virus replication by small interfering RNAs in mammalian cells. *J Virol.* (2004) 78:7523–7. doi: 10.1128/JVI.78.14.7523-7527.2004
112. Wu C-J, Huang H-W, Liu C-Y, Hong C-F, Chan Y-L. Inhibition of SARS-CoV replication by siRNA. *Antiviral Res.* (2005) 65:45–8. doi: 10.1016/j.antiviral.2004.09.005
113. Zheng B-j, Guan Y, Tang Q, Du C, Xie FY, He M-L, et al. Prophylactic and therapeutic effects of small interfering RNA targeting SARS coronavirus. *Antiviral Ther.* (2004) 9:365–74.
114. Liu Y-C, Huang V, Chao T-C, Hsiao C-D, Lin A, Chang M-F, et al. Screening of drugs by FRET analysis identifies inhibitors of SARS-CoV 3CL protease. *Biochem Biophys Res Commun.* (2005) 333:194–9. doi: 10.1016/j.bbrc.2005.05.095
115. Barnard DL, Kumaki Y. Recent developments in anti-severe acute respiratory syndrome coronavirus chemotherapy. *Fut Virol.* (2011) 6:615–31. doi: 10.2217/fvl.11.33
116. Barretto N, Jukneliene D, Ratia K, Chen Z, Mesecar AD, Baker SC. Deubiquitinating activity of the SARS-CoV papain-like protease. *Nidoviruses.* (2006) 581:37–41. doi: 10.1007/978-0-387-33012-9_5
117. Lindner HA, Lytvyn V, Qi H, Lachance P, Ziomek E, Ménard R. Selectivity in ISG15 and ubiquitin recognition by the SARS coronavirus papain-like protease. *Arch Biochem Biophys.* (2007) 466:8–14. doi: 10.1016/j.abb.2007.07.006
118. Yang H, Bartlam M, Rao Z. Drug design targeting the main protease, the Achilles' heel of coronaviruses. *Curr Pharma Design.* (2006) 12:4573–90. doi: 10.2174/138161206779010369
119. Liang P-H. Characterization and inhibition of SARS-coronavirus main protease. *Curr Top Med Chem.* (2006) 6:361–76. doi: 10.2174/156802606776287090
120. Graziano V, McGrath WJ, DeGruccio AM, Dunn JJ, Mangel WF. Enzymatic activity of the SARS coronavirus main proteinase dimer. *FEBS Lett.* (2006) 580:2577–83. doi: 10.1016/j.febslet.2006.04.004
121. Imbert I, Guillemot JC, Bourhis JM, Bussetta C, Coutard B, Egloff MP, et al. A second, non-canonical RNA-dependent RNA polymerase in SARS Coronavirus. *Embo J.* (2006) 25:4933–42. doi: 10.1038/sj.emboj.7601368
122. Te Velthuis AJ, van den Worm SH, Sims AC, Baric RS, Snijder EJ, van Hemert MJ. Zn2+ inhibits coronavirus and arterivirus RNA polymerase activity *in vitro* and zinc ionophores block the replication of these viruses in cell culture. *PLoS Pathog.* (2010) 6:e1001176. doi: 10.1371/journal.ppat.1001176
123. Prentice E, McAuliffe J, Lu X, Subbarao K, Denison MR. Identification and characterization of severe acute respiratory syndrome coronavirus replicase proteins. *J Virol.* (2004) 78:9977–86. doi: 10.1128/JVI.78.18.9977-9986.2004
124. Fan Z, Peng K, Tan X, Yin B, Dong X, Qiu F, et al. Molecular cloning, expression, and purification of SARS-CoV nsp13. *Prot Exp Purif.* (2005) 41:235–40. doi: 10.1016/j.pep.2004.08.003
125. Ivanov KA, Thiel V, Dobbe JC, van der Meer Y, Snijder EJ, Ziebuhr J. Multiple enzymatic activities associated with severe acute respiratory syndrome coronavirus helicase. *J Virol.* (2004) 78:5619–32. doi: 10.1128/JVI.78.11.5619-5632.2004
126. Yang N, Tanner JA, Wang Z, Huang J-D, Zheng B-J, Zhu N, et al. Inhibition of SARS coronavirus helicase by bismuth complexes. *Chem Commun.* (2007) 4413–15. doi: 10.1039/b709515e
127. Yang N, Tanner JA, Zheng BJ, Watt RM, He ML, Lu LY, et al. Bismuth complexes inhibit the SARS coronavirus. *Angew Chem Int Ed.* (2007) 46:6464–8. doi: 10.1002/anie.200701021
128. ter Meulen J, Bakker AB, van den Brink EN, Weverling GJ, Martina BE, Haagmans BL, et al. Human monoclonal antibody as prophylaxis for SARS coronavirus infection in ferrets. *Lancet.* (2004) 363:2139–41. doi: 10.1016/S0140-6736(04)16506-9
129. Kumaki Y, Ennis J, Rahbar R, Turner JD, Wandersee MK, Smith AJ, et al. Single-dose intranasal administration with mDEF201 (adenovirus vectored mouse interferon-alpha) confers protection from mortality in a lethal SARS-CoV BALB/c mouse model. *Antiviral Res.* (2011) 89:75–82. doi: 10.1016/j.antiviral.2010.11.007
130. Kumaki Y, Day CW, Wandersee MK, Schow BP, Madsen JS, Grant D, et al. Interferon alfacon 1 inhibits SARS-CoV infection in human bronchial epithelial Calu-3 cells. *Biochem Biophys Res Commun.* (2008) 371:110–3. doi: 10.1016/j.bbrc.2008.04.006
131. Day CW, Baric R, Cai SX, Frieman M, Kumaki Y, Morrey JD, et al. A new mouse-adapted strain of SARS-CoV as a lethal model for evaluating antiviral agents *in vitro* and *in vivo*. *Virology.* (2009) 395:210–22. doi: 10.1016/j.virol.2009.09.023

132. Zhu X, Nishimura F, Sasaki K, Fujita M, Dusak JE, Eguchi J, et al. Toll like receptor-3 ligand poly-ICLC promotes the efficacy of peripheral vaccinations with tumor antigen-derived peptide epitopes in murine CNS tumor models. *J Transl Med.* (2007) 5:10. doi: 10.1186/1479-5876-5-10
133. Xiao Y, Ma Q, Restle T, Shang W, Svergun DI, Ponnusamy R, et al. Nonstructural proteins 7 and 8 of feline coronavirus form a 2: 1 heterotrimer that exhibits primer-independent RNA polymerase activity. *J Virol.* (2012) 86:4444–54. doi: 10.1128/JVI.06635-11
134. Li S, Zhao Q, Zhang Y, Zhang Y, Bartlam M, Li X, et al. New nsp8 isoform suggests mechanism for tuning viral RNA synthesis. *Protein Cell.* (2010) 1:198–204. doi: 10.1007/s13238-010-0028-8
135. Gao J, Tian Z, Yang X. Breakthrough: chloroquine phosphate has shown apparent efficacy in treatment of COVID-19 associated pneumonia in clinical studies. *Biosci Trends*. (2020) 14:72–3. doi: 10.5582/bst.2020.01047
136. Cao B, Wang Y, Wen D, Liu W, Wang J, Fan G, et al. A trial of lopinavir–ritonavir in adults hospitalized with severe Covid-19. *N Engl J Med*. (2020) 382:1787–99. doi: 10.1056/NEJMoa2001282
137. Andersen KG, Rambaut A, Lipkin WI, Holmes EC, Garry RF. The proximal origin of SARS-CoV-2. *Nat Med.* (2020) 26:450–2. doi: 10.1038/s41591-020-0820-9
138. Menachery VD, Dinnon KH, Yount BL, McAnarney ET, Gralinski LE, Hale A, et al. Trypsin treatment unlocks barrier for zoonotic bat coronavirus infection. *J Virol.* (2020) 94:e01774-19. doi: 10.1128/JVI.01774-19
139. Liu W, Fontanet A, Zhang P-H, Zhan L, Xin Z-T, Baril L, et al. Two-year prospective study of the humoral immune response of patients with severe acute respiratory syndrome. *J Infect Dis.* (2006) 193:792–5. doi: 10.1086/500469
140. Vickers C, Hales P, Kaushik V, Dick L, Gavin J, Tang J, et al. Hydrolysis of biological peptides by human angiotensin-converting enzyme-related carboxypeptidase. *J Biol Chem.* (2002) 277:14838–43. doi: 10.1074/jbc.M200581200
141. Rice GI, Thomas DA, Grant PJ, Turner AJ, Hooper NM. Evaluation of angiotensin-converting enzyme (ACE), its homologue ACE2 and neprilysin in angiotensin peptide metabolism. *Biochem J.* (2004) 383:45–51. doi: 10.1042/BJ20040634
142. Turner AJ, Hiscox JA, Hooper NM. ACE2: from vasopeptidase to SARS virus receptor. *Trends Pharmacol Sci.* (2004) 25:291–4. doi: 10.1016/j.tips.2004.04.001
143. Rella M, Rushworth CA, Guy JL, Turner AJ, Langer T, Jackson RM. Structure-based pharmacophore design and virtual screening for novel angiotensin converting enzyme 2 inhibitors. *J Chem Inform Model.* (2006) 46:708–16. doi: 10.1021/ci0503614
144. Patel VB, Zhong J-C, Grant MB, Oudit GY. Role of the ACE2/angiotensin 1–7 axis of the renin–angiotensin system in heart failure. *Circ Res.* (2016) 118:1313–26. doi: 10.1161/CIRCRESAHA.116.307708
145. Warner FJ, Lew RA, Smith AI, Lambert DW, Hooper NM, Turner AJ. Angiotensin-converting enzyme 2 (ACE2), but not ACE, is preferentially localized to the apical surface of polarized kidney cells. *J Biol Chem.* (2005) 280:39353–62. doi: 10.1074/jbc.M508914200
146. Xia H, Suda S, Bindom S, Feng Y, Gurley S. ACE2-mediated reduction of oxidative stress in the central nervous system is associated with improvement of autonomic function. *PLoS ONE.* (2011) 6:e22682. doi: 10.1371/journal.pone.0022682
147. Guan W-J, Ni Z-Y, Hu Y, Liang W-H, Ou C-Q, He J-X, et al. Clinical characteristics of coronavirus disease 2019 in China. *N Engl J Med*. (2020) 18:1708–20. doi: 10.1101/2020.02.06.20020974
148. Xu X, Chen P, Wang J, Feng J, Zhou H, Li X, et al. Evolution of the novel coronavirus from the ongoing Wuhan outbreak and modeling of its spike protein for risk of human transmission. *Sci China Life Sci.* (2020) 63:457–60. doi: 10.1007/s11427-020-1637-5
149. Diaz JH. Hypothesis: angiotensin-converting enzyme inhibitors and angiotensin receptor blockers may increase the risk of severe COVID-19. *J Travel Med.* (2020) 27. doi: 10.1093/jtm/taaa041
150. Barnard D, Day C, Wandersee M, Kumaki Y, Salazar A. Evaluation of interferon inducers, ribavirin and mouse hyperimmune serum in a pathogenesis/lethal mouse model using a mouse-adapted SARS-CoV. *Antiviral Res.* (2008) 2:A20. doi: 10.1016/j.antiviral.2008.01.025
151. Barnard DL, Day CW, Bailey K, Heiner M, Montgomery R, Lauridsen L, et al. Enhancement of the infectivity of SARS-CoV in BALB/c mice by IMP dehydrogenase inhibitors, including ribavirin. *Antiviral Res.* (2006) 71:53–63. doi: 10.1016/j.antiviral.2006.03.001
152. Barnard DL, Day CW, Bailey K, Heiner M, Montgomery R, Lauridsen L, et al. Is the anti-psychotic, 10-(3-(dimethylamino) propyl) phenothiazine (promazine), a potential drug with which to treat SARS infections?: Lack of efficacy of promazine on SARS-CoV replication in a mouse model. *Antiviral Res.* (2008) 79:105–13. doi: 10.1016/j.antiviral.2007.12.005
153. Reghunathan R, Jayapal M, Hsu L-Y, Chng H-H, Tai D, Leung BP, et al. Expression profile of immune response genes in patients with severe acute respiratory syndrome. *BMC Immunol.* (2005) 6:2. doi: 10.1186/1471-2172-6-2
154. Eroshenko N, Gill T, Keaveney MK, Church GM, Trevejo JM, Rajaniemi H. Implications of antibody-dependent enhancement of infection for SARS-CoV-2 countermeasures. *Nat Biotechnol.* (2020) 38:789–91. doi: 10.1038/s41587-020-0577-1
155. Channappanavar R, Perlman S. Pathogenic human coronavirus infections: causes and consequences of cytokine storm and immunopathology. *Semin Immunopathol.* (2017) 39:529–39. doi: 10.1007/s00281-017-0629-x
156. Tseng C-T, Sbrana E, Iwata-Yoshikawa N, Newman PC, Garron T, Atmar RL, et al. Immunization with SARS coronavirus vaccines leads to pulmonary immunopathology on challenge with the SARS virus. *PLoS ONE.* (2012) 7:e35421. doi: 10.1371/journal.pone.0035421
157. Spiegel M, Pichlmair A, Martínez-Sobrido L, Cros J, García-Sastre A, Haller O, et al. Inhibition of beta interferon induction by severe acute respiratory syndrome coronavirus suggests a two-step model for activation of interferon regulatory factor 3. *J Virol.* (2005) 79:2079–86. doi: 10.1128/JVI.79.4.2079-2086.2005
158. Law HK, Cheung CY, Ng HY, Sia SF, Chan YO, Luk W, et al. Chemokine up-regulation in sars-coronavirus–infected, monocyte-derived human dendritic cells. *Blood.* (2005) 106:2366–74. doi: 10.1182/blood-2004-10-4166
159. Surjit M, Liu B, Jameel S, Chow VT, Lal SK. The SARS coronavirus nucleocapsid protein induces actin reorganization and apoptosis in COS-1 cells in the absence of growth factors. *Biochem J.* (2004) 383:13–8. doi: 10.1042/BJ20040984
160. Law PT, Wong C-H, Au TC, Chuck C-P, Kong S-K, Chan PK, et al. The 3a protein of severe acute respiratory syndrome-associated coronavirus induces apoptosis in Vero E6 cells. *J General Virol.* (2005) 86:1921–30. doi: 10.1099/vir.0.80813-0
161. Tan Y-J, Fielding BC, Goh P-Y, Shen S, Tan TH, Lim SG, et al. Overexpression of 7a, a protein specifically encoded by the severe acute respiratory syndrome coronavirus, induces apoptosis via a caspase-dependent pathway. *J Virol.* (2004) 78:14043–7. doi: 10.1128/JVI.78.24.14043-14047.2004
162. Yuan X, Shan Y, Zhao Z, Chen J, Cong Y. G0/G1 arrest and apoptosis induced by SARS-CoV 3b protein in transfected cells. *Virol J.* (2005) 2:66. doi: 10.1186/1743-422X-2-66
163. Li T, Qiu Z, Zhang L, Han Y, He W, Liu Z, et al. Significant changes of peripheral T lymphocyte subsets in patients with severe acute respiratory syndrome. *J Infect Dis.* (2004) 189:648–51. doi: 10.1086/381535
164. Dhodapkar KM, Banerjee D, Connolly J, Kukreja A, Matayeva E, Veri MC, et al. Selective blockade of the inhibitory Fcγ receptor (FcγRIIB) in human dendritic cells and monocytes induces a type I interferon response program. *J Exp Med.* (2007) 204:1359–69. doi: 10.1084/jem.20062545
165. Chan KR, Zhang SL-X, Tan HC, Chan YK, Chow A, Lim APC, et al. Ligation of Fc gamma receptor IIB inhibits antibody-dependent enhancement of dengue virus infection. *Proc Natl Acad Sci USA.* (2011) 108:12479–84. doi: 10.1073/pnas.1106568108
166. Nagelkerke SQ, Kuijpers TW. Immunomodulation by IVIg and the role of Fc-gamma receptors: classic mechanisms of action after all? *Front Immunol.* (2015) 5:674. doi: 10.3389/fimmu.2014.00674
167. Lee C. Therapeutic modulation of virus-induced oxidative stress via the Nrf2-dependent antioxidative pathway. *Oxid Med Cell Longev*. (2018) 2018:6208067. doi: 10.1155/2018/6208067
168. Isaguliants M, Smirnova O, Ivanov AV, Kilpelainen A, Kuzmenko Y, Petkov S, et al. Oxidative stress induced by HIV-1 reverse transcriptase modulates the enzyme's performance in gene immunization. *Hum Vaccines Immunother.* (2013) 9:2111–9. doi: 10.4161/hv.25813

169. Winterbourn CC, Hampton MB. Thiol chemistry and specificity in redox signaling. *Free Radic Biol Med.* (2008) 45:549–61. doi: 10.1016/j.freeradbiomed.2008.05.004
170. Cho H-Y, Imani F, Miller-DeGraff L, Walters D, Melendi GA, Yamamoto M, et al. Antiviral activity of Nrf2 in a murine model of respiratory syncytial virus disease. *Am J Respir Crit Care Med.* (2009) 179:138–50. doi: 10.1164/rccm.200804-535OC
171. Lee Y-H, Lai C-L, Hsieh S-H, Shieh C-C, Huang L-M, Wu-Hsieh BA. Influenza A virus induction of oxidative stress and MMP-9 is associated with severe lung pathology in a mouse model. *Virus Res.* (2013) 178:411–22. doi: 10.1016/j.virusres.2013.09.011
172. Okuda M, Li K, Beard MR, Showalter LA, Scholle F, Lemon SM, et al. Mitochondrial injury, oxidative stress, and antioxidant gene expression are induced by hepatitis C virus core protein. *Gastroenterology.* (2002) 122:366–75. doi: 10.1053/gast.2002.30983
173. Olagnier D, Peri S, Steel C, van Montfoort N, Chiang C, Beljanski V, et al. Cellular oxidative stress response controls the antiviral and apoptotic programs in dengue virus-infected dendritic cells. *PLoS Pathog.* (2014) 10:e1004566. doi: 10.1371/journal.ppat.1004566
174. Fukuyama S, Kawaoka Y. The pathogenesis of influenza virus infections: the contributions of virus and host factors. *Curr Opin Immunol.* (2011) 23:481–6. doi: 10.1016/j.coi.2011.07.016
175. Hosakote YM, Jantzi PD, Esham DL, Spratt H, Kurosky A, Casola A, et al. Viral-mediated inhibition of antioxidant enzymes contributes to the pathogenesis of severe respiratory syncytial virus bronchiolitis. *Am J Respir Crit Care Med.* (2011) 183:1550–60. doi: 10.1164/rccm.201010-1755OC
176. Komaravelli N, Ansar M, Garofalo RP, Casola A. Respiratory syncytial virus induces NRF2 degradation through a promyelocytic leukemia protein-ring finger protein 4 dependent pathway. *Free Radic Biol Med.* (2017) 113:494–504. doi: 10.1016/j.freeradbiomed.2017.10.380
177. Staitieh BS, Ding L, Neveu WA, Spearman P, Guidot DM, Fan X. HIV-1 decreases Nrf2/ARE activity and phagocytic function in alveolar macrophages. *J Leukoc Biol.* (2017) 102:517–25. doi: 10.1189/jlb.4A0616-282RR
178. Qiang W, Kuang X, Liu J, Liu N, Scofield VL, Reid AJ, et al. Astrocytes survive chronic infection and cytopathic effects of the ts1 mutant of the retrovirus Moloney murine leukemia virus by upregulation of antioxidant defenses. *J Virol.* (2006) 80:3273–84. doi: 10.1128/JVI.80.7.3273-3284.2006
179. Carvajal-Yepes M, Himmelsbach K, Schaedler S, Ploen D, Krause J, Ludwig L, et al. Hepatitis C virus impairs the induction of cytoprotective Nrf2 target genes by delocalization of small Maf proteins. *J Biol Chem.* (2011) 286:8941–51. doi: 10.1074/jbc.M110.186684
180. Machida K, Cheng KT-H, Lai C-K, Jeng K-S, Sung VM-H, Lai MM. Hepatitis C virus triggers mitochondrial permeability transition with production of reactive oxygen species, leading to DNA damage and STAT3 activation. *J Virol.* (2006) 80:7199–207. doi: 10.1128/JVI.00321-06
181. Komaravelli N, Tian B, Ivanciuc T, Mautemps N, Brasier AR, Garofalo RP, et al. Respiratory syncytial virus infection down-regulates antioxidant enzyme expression by triggering deacetylation-proteasomal degradation of Nrf2. *Free Radic Biol Med.* (2015) 88:391–403. doi: 10.1016/j.freeradbiomed.2015.05.043
182. Johnson B, Li J, Adhikari J, Edwards MR, Zhang H, Schwarz T, et al. Dimerization controls Marburg virus VP24-dependent modulation of host antioxidative stress responses. *J Mol Biol.* (2016) 428:3483–94. doi: 10.1016/j.jmb.2016.07.020
183. Page A, Volchkova VA, Reid SP, Mateo M, Bagnaud-Baule A, Nemirov K, et al. Marburgvirus hijacks nrf2-dependent pathway by targeting nrf2-negative regulator keap1. *Cell Rep.* (2014) 6:1026–36. doi: 10.1016/j.celrep.2014.02.027
184. Cheng Y-L, Lin Y-S, Chen C-L, Tsai T-T, Tsai C-C, Wu Y-W, et al. Activation of Nrf2 by the dengue virus causes an increase in CLEC5A, which enhances TNF-α production by mononuclear phagocytes. *Sci Rep.* (2016) 6:32000. doi: 10.1038/srep32000
185. Kesic MJ, Simmons SO, Bauer R, Jaspers I. Nrf2 expression modifies influenza A entry and replication in nasal epithelial cells. *Free Radic Biol Med.* (2011) 51:444–453. doi: 10.1016/j.freeradbiomed.2011.04.027
186. Kosmider B, Messier EM, Janssen WJ, Nahreini P, Wang J, Hartshorn KL, et al. Nrf2 protects human alveolar epithelial cells against injury induced by influenza A virus. *Respir Res.* (2012) 13:43. doi: 10.1186/1465-9921-13-43
187. Chandran K, Sullivan NJ, Felbor U, Whelan SP, Cunningham JM. Endosomal proteolysis of the Ebola virus glycoprotein is necessary for infection. *Science.* (2005) 308:1643–5. doi: 10.1126/science.1110656
188. Matsuyama S, Taguchi F. Two-step conformational changes in a coronavirus envelope glycoprotein mediated by receptor binding and proteolysis. *J Virol.* (2009) 83:11133–41. doi: 10.1128/JVI.00959-09
189. Simmons G, Reeves JD, Rennekamp AJ, Amberg SM, Piefer AJ, Bates P. Characterization of severe acute respiratory syndrome-associated coronavirus (SARS-CoV) spike glycoprotein-mediated viral entry. *Proc Natl Acad Sci USA.* (2004) 101:4240–5. doi: 10.1073/pnas.0306446101
190. Böttcher E, Matrosovich T, Beyerle M, Klenk H-D, Garten W, Matrosovich M. Proteolytic activation of influenza viruses by serine proteases TMPRSS2 and HAT from human airway epithelium. *J Virol.* (2006) 80:9896–8. doi: 10.1128/JVI.01118-06
191. Jiang Y, Scofield VL, Yan M, Qiang W, Liu N, Reid AJ, et al. Retrovirus-induced oxidative stress with neuroimmunodegeneration is suppressed by antioxidant treatment with a refined monosodium α-luminol (Galavit). *J Virol.* (2006) 80:4557–69. doi: 10.1128/JVI.80.9.4557-4569.2006
192. Zhang H-S, Wu T-C, Sang W-W, Ruan Z. EGCG inhibits Tat-induced LTR transactivation: role of Nrf2, AKT, AMPK signaling pathway. *Life Sci.* (2012) 90:747–54. doi: 10.1016/j.lfs.2012.03.013
193. Zhang HS, Chen XY, Wu TC, Zhang FJ. Tanshinone II A inhibits tat-induced HIV-1 transactivation through redox-regulated AMPK/Nampt pathway. *J Cell Physiol.* (2014) 229:1193–201. doi: 10.1002/jcp.24552
194. Fan X, Staitieh BS, Jensen JS, Mould KJ, Greenberg JA, Joshi PC, et al. Activating the Nrf2-mediated antioxidant response element restores barrier function in the alveolar epithelium of HIV-1 transgenic rats. *Am J Physiol Lung Cell Mol Physiol.* (2013) 305:L267–77. doi: 10.1152/ajplung.00288.2012
195. Chen W-C, Tseng C-K, Lin C-K, Wang S-N, Wang W-H, Hsu S-H, et al. Lucidone suppresses dengue viral replication through the induction of heme oxygenase-1. *Virulence.* (2018) 9:588–603. doi: 10.1080/21505594.2017.1421893
196. Chen J-X, Xue H-J, Ye W-C, Fang B-H, Liu Y-H, Yuan S-H, et al. Activity of andrographolide and its derivatives against influenza virus *in vivo* and *in vitro*. *Biol Pharma Bull.* (2009) 32:1385–91. doi: 10.1248/bpb.32.1385
197. Youn GS, Kwon D-J, Ju SM, Rhim H, Bae YS, Choi SY, et al. Celastrol ameliorates HIV-1 Tat-induced inflammatory responses via NF-kappaB and AP-1 inhibition and heme oxygenase-1 induction in astrocytes. *Toxicol Appl Pharmacol.* (2014) 280:42–52. doi: 10.1016/j.taap.2014.07.010
198. Noah TL, Zhang H, Zhou H, Glista-Baker E, Müller L, Bauer RN, et al. Effect of broccoli sprouts on nasal response to live attenuated influenza virus in smokers: a randomized, double-blind study. *PLoS ONE.* (2014) 9:e98671. doi: 10.1371/journal.pone.0098671
199. Shoji M, Arakaki Y, Esumi T, Kohnomi S, Yamamoto C, Suzuki Y, et al. Bakuchiol is a phenolic isoprenoid with novel enantiomer-selective anti-influenza A virus activity involving Nrf2 activation. *J Biol Chem.* (2015) 290:28001–17. doi: 10.1074/jbc.M115.669465
200. Yin J, Ma L, Wang H, Yan H, Hu J, Jiang W, et al. Chinese herbal medicine compound Yi-Zhi-Hao pellet inhibits replication of influenza virus infection through activation of heme oxygenase-1. *Acta Pharma Sin B.* (2017) 7:630–7. doi: 10.1016/j.apsb.2017.05.006
201. Dai J, Gu L, Su Y, Wang Q, Zhao Y, Chen X, et al. Inhibition of curcumin on influenza A virus infection and influenzal pneumonia via oxidative stress, TLR2/4, p38/JNK MAPK and NF-κB pathways. *Int Immunopharmacol.* (2018) 54:177–87. doi: 10.1016/j.intimp.2017.11.009
202. Snitsarev V, Young MN, Miller RM, Rotella DP. The spectral properties of (-)-epigallocatechin 3-O-gallate (EGCG) fluorescence in different solvents: Dependence on solvent polarity. *PLoS ONE.* (2013) 8:e79834. doi: 10.1371/journal.pone.0079834
203. Ahn Y-H, Hwang Y, Liu H, Wang XJ, Zhang Y, Stephenson KK, et al. Electrophilic tuning of the chemoprotective natural product sulforaphane. *Proc Natl Acad Sci USA.* (2010) 107:9590–5. doi: 10.1073/pnas.1004104107
204. Wang Y, Yan J, Li S, Cai X, Wang W, Luo K, et al. Pharmacokinetics and tissue distribution study of tanshinone IIA after oral administration of Bushen

Huoxue Qubi granules to rats with blood stasis syndrome. *Pharmacogn Mag.* (2014) 10:285. doi: 10.4103/0973-1296.137369
205. Cascão R, Fonseca JE, Moita LF. Celastrol: a spectrum of treatment opportunities in chronic diseases. *Front Med.* (2017) 4:69. doi: 10.3389/fmed.2017.00069
206. Alalaiwe A, Hung C-F, Leu Y-L, Tahara K, Chen H-H, Hu K-Y, et al. The active compounds derived from *Psoralea corylifolia* for photochemotherapy against psoriasis-like lesions: the relationship between structure and percutaneous absorption. *Eur J Pharma Sci.* (2018) 124:114–26. doi: 10.1016/j.ejps.2018.08.031
207. He Y-W, Dong C-Z, Zhao J-Y, Ma L-L, Li Y-H, Aisa HA. 1, 2, 3-Triazole-containing derivatives of rupestonic acid: click-chemical synthesis and antiviral activities against influenza viruses. *Eur J Med Chem*. (2014) 76:245–55. doi: 10.1016/j.ejmech.2014.02.029
208. Amalraj A, Pius A, Gopi S, Gopi S. Biological activities of curcuminoids, other biomolecules from turmeric and their derivatives–a review. *J Tradit Complement Med.* (2017) 7:205–33. doi: 10.1016/j.jtcme.2016.05.005
209. Drăgoi A-L. Potent NRF2-activating dietary supplements (like resveratrol, curcumin, sulforaphane, "Asea redox supplement" [ARS]) should be clinically tested as adjuvants in all types of medium and severe cases of aggressive respiratory viral infections (including Influenza A/B/C, SARS, MERS, COVID-19, measles, avian influenza etc.) based on their extrapolated cytoprotective antioxidant effects (especially on vital organs), including the cytoprotection offered by ARS on the cardiac muscle of DMD patients which can be extrapolated to the lungs - A very short medical communication. (2019). doi: 10.13140/RG.2.2.33764.12163G.2.2.33764.12163
210. Lu R, Zhao X, Li J, Niu P, Yang B, Wu H, et al. Genomic characterisation and epidemiology of 2019 novel coronavirus: implications for virus origins and receptor binding. *Lancet.* (2020) 395:565–74. doi: 10.1016/S0140-6736(20)30251-8
211. Chen H, Du Q. Potential natural compounds for preventing 2019-nCoV infection. *Europe PMC [preprint].* (2020). doi: 10.20944/preprints202001.0358.v3
212. Lin C-W, Tsai F-J, Tsai C-H, Lai C-C, Wan L, Ho T-Y, et al.-Chao DL. Anti-SARS coronavirus 3C-like protease effects of *Isatis indigotica* root and plant-derived phenolic compounds. *Antiviral Res.* (2005) 68:36–42. doi: 10.1016/j.antiviral.2005.07.002
213. Cheng LP, Zheng WK, Li M, Huang J, Bao SZ, Xu Q, et al. Citrus fruits are rich in flavonoids for immunoregulation and potential targeting ACE2. *Preprints*. (2020) 2020:1–13.
214. Wen C-C, Kuo Y-H, Jan J-T, Liang P-H, Wang S-Y, Liu H-G, et al. Specific plant terpenoids and lignoids possess potent antiviral activities against severe acute respiratory syndrome coronavirus. *J Med Chem.* (2007) 50:4087–95. doi: 10.1021/jm070295s
215. O'Keefe BR, Giomarelli B, Barnard DL, Shenoy SR, Chan PK, McMahon JB, et al. Broad-spectrum *in vitro* activity and *in vivo* efficacy of the antiviral protein griffithsin against emerging viruses of the family Coronaviridae. *J Virol.* (2010) 84:2511–21. doi: 10.1128/JVI.02322-09
216. Kim DW, Seo KH, Curtis-Long MJ, Oh KY, Oh J-W, Cho JK, et al. Phenolic phytochemical displaying SARS-CoV papain-like protease inhibition from the seeds of Psoralea corylifolia. *J Enzyme Inhibit Med Chem.* (2014) 29:59–63. doi: 10.3109/14756366.2012.753591
217. Moghaddam E, Teoh B-T, Sam S-S, Lani R, Hassandarvish P, Chik Z, et al. Baicalin, a metabolite of baicalein with antiviral activity against dengue virus. *Sci Rep.* (2014) 4:5452. doi: 10.1038/srep05452
218. Liu Q, Li X, Ouyang X, Chen D. Dual effect of glucuronidation of a Pyrogallol-type phytophenol antioxidant: a comparison between scutellarein and scutellarin. *Molecules.* (2018) 23:3225. doi: 10.3390/molecules23123225
219. Fushiya S, Takahashi K, Nakatsuyama S, Sato Y, Nozoe S, Takagi S-I. Co-occurrence of nicotianamine and avenic acids in *Avena sativa* and *Oryza sativa*. *Phytochemistry.* (1982) 21:1907–8. doi: 10.1016/0031-9422(82)83012-4
220. Kristensen I, Larsen PO. Azetidine-2-carboxylic acid derivatives from seeds of *Fagus silvatica* L. and a revised structure for nicotianamine. *Phytochemistry.* (1974) 13:2791–8. doi: 10.1016/0031-9422(74)80243-8
221. Hordyjewska A, Ostapiuk A, Horecka A, Kurzepa J. Betulin and betulinic acid: triterpenoids derivatives with a powerful biological potential. *Phytochem Rev.* (2019) 18:929–51. doi: 10.1007/s11101-019-09623-1
222. Xue J, Hoorelbeke B, Kagiampakis I, Demeler B, Balzarini J, LiWang PJ. The griffithsin dimer is required for high-potency inhibition of HIV-1: evidence for manipulation of the structure of gp120 as part of the griffithsin dimer mechanism. *Antimicrob Agents Chemother.* (2013) 57:3976–89. doi: 10.1128/AAC.00332-13

Conflict of Interest: GA is employed by GALLY International Biomedical Research & Consulting LLC.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Public Health

ORIGINAL RESEARCH
published: 22 September 2020
doi: 10.3389/fpubh.2020.570543

Antibody Profiling of COVID-19 Patients in an Urban Low-Incidence Region in Northern Germany

Werner Solbach[1,2*†], *Julia Schiffner*[2†], *Insa Backhaus*[2,3], *David Burger*[4], *Ralf Staiger*[5], *Bettina Tiemer*[6], *Andreas Bobrowski*[6], *Timothy Hutchings*[4] and *Alexander Mischnik*[2]

[1] Center for Infection and Inflammation Research, University of Lübeck, Lübeck, Germany, [2] Health Protection Authority, Lübeck, Germany, [3] Department of Public Health and Infectious Diseases, Sapienza University of Rome, Rome, Italy, [4] Municipal Statistics Department, Lübeck, Germany, [5] Gemeinschaftspraxis Huextertor, Lübeck, Germany, [6] Laboraerztliche Gemeinschaftspraxis Lübeck, Lübeck, Germany

A vast majority of COVID-19 cases present with mild or moderate symptoms. The study region is in an urban and well-defined environment in a low-incidence region in Northern Germany. In the present study, we explored the dynamics of the antibody response with respect to onset, level and duration in patients with confirmed SARS-CoV-2 infection. Anti-SARS-CoV-2 IgG and IgA were detected by automated enzyme-linked immunosorbent assay (ELISA) of SARS-CoV-2 infected patients monitored by the Health Protection Authority. This explorative monocentric study shows IgA and IgG antibody profiles from 118 patients with self-reported mild to moderate, or no COVID-19 related symptoms after laboratory-confirmed infection with SARS-CoV-2. We found that 21.7% and 18.1% of patients were seronegative for IgA or IgG, respectively. Clinically, most of the seronegative patients showed no to only moderate symptoms. With regard to antibody profiling 82% of all patients developed sustainable antibodies (IgG) and 78% (IgA) 3 weeks or later after the infection. Our data indicate that antibody-positivity is a useful indicator of a previous SARS-CoV-2 infection. Negative antibodies do not rule out SARS-CoV-2 infection. Future studies are needed to determine the functionality of the antibodies in terms of neutralization capacity leading to personal protection and prevention ability to transmit the virus as well as to protect after vaccination.

Keywords: COVID-19, SARS-CoV-2, immunoglobulin, IgG, IgA, seroprevalence, herd immunity

OPEN ACCESS

Edited by:
Zhongheng Zhang,
Sir Run Run Shaw Hospital, China

Reviewed by:
Genevieve Fouda,
Duke University, United States
Takashi Hashimoto,
Osaka City University, Japan
Jing Yuan,
Children's Hospital of Capital Institute of Pediatrics, China
Justin Pollara,
Duke University School of Medicine, United States
Juanjuan Zhao,
Shenzhen Third People's Hospital, China
Manuel Serrano,
San Carlos University Clinical Hospital, Spain

***Correspondence:**
Werner Solbach
werner.solbach@uni-luebeck.de

*These authors have contributed equally to this work

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Public Health

Received: *08 June 2020*
Accepted: *27 August 2020*
Published: *22 September 2020*

Citation:
Solbach W, Schiffner J, Backhaus I, Burger D, Staiger R, Tiemer B, Bobrowski A, Hutchings T and Mischnik A (2020) Antibody Profiling of COVID-19 Patients in an Urban Low-Incidence Region in Northern Germany. Front. Public Health 8:570543. doi: 10.3389/fpubh.2020.570543

INTRODUCTION

The novel severe acute respiratory syndrome coronavirus (SARS-CoV-2) causes a respiratory disease, known as COVID-19 (1). On December 31, 2019, Chinese officials reported a cluster of cases of pneumonia in Wuhan, China. The infection was quickly qualified as epidemic (2). As of January 30, 2020, it was announced a public health emergency of international concern. As of March 11, 2020 WHO officially declared the epidemic a pandemic (World Health Organization, Director-General's Opening Remarks at the MediaBriefing on COVID-19 - 11 March 2020). Early reports from China and Italy indicated that SARS-CoV-2 causes illness of varying degrees (3). A vast majority of COVID-19 cases present with mild or moderate symptoms ranging from fatigue, sore throat, cough and fever to a more severe disease course including acute respiratory distress syndrome and septic shock (4, 5). The infection can spread easily as the virus is able to transmit during the

presymptomatic or asymptomatic phase of infection (6, 7). In Germany, the first COVID-19 case was detected on January 27, 2020 and spread rapidly around the country (8, 9). On February 28, 2020, Germany's national Public Health Institute (Robert Koch Institute [RKI]) rated the risk of the COVID-19 pandemic for the population in Germany as "low to moderate," which was then revised to "high" (March 17, 2020) and to "very high" for risk groups (March 26,2020)[1].

The core basis for the management of the outbreak is the early detection of SARS-CoV-2 in respiratory specimens (nasopharyngeal swabs) from patients presenting with clinical signs such as fever, dry cough or shortness of breath or in asymptomatic persons with close contact to a laboratory confirmed COVID-19 case. People who have a cumulative face-to-face contact with a confirmed case for ≥15 min, direct contact with secretions or body fluids of a patient with confirmed COVID-19 disease, or, in the case of health-care workers, work within 2 m of a patient with confirmed COVID-19 disease without personal protective equipment are at high risk for infection (10). The gold standard for SARS-CoV-2-detection is a specific polymerase chain reaction (PCR) testing from a nasopharyngeal swab, sputum, or broncoalveolar lavage (11). Recently, commercial assays for serological analysis of specific COVID-19 antibodies became available (12, 13). From a public health perspective it is an easy to establish and cost effective laboratory-based screening strategy that may assist in rapid case detection and surveillance and ultimately in a better understanding of this epidemic (10). Since there is no specific medical treatment or a vaccine available at present, it is crucial that sufficient herd immunity will develop in the population to interrupt uncontrollable transmission of the virus. Like in other coronaviruses, it is likely that neutralizing antibodies are central to the development of herd immunity to SARS-CoV-2. Therefore, insight into the development of immunity is pertinent for future guidance of preventive measures. In addition, antibody levels may give information on whether patients with COVID-19 infection are immune to re-infection. However, given that SARS-CoV-2 is a newly emerging virus, the antibody response remains largely unknown.

At present, different investigations are ongoing to get insight in seroprevalence of COVID-19 infection in Germany and Europe. Many researchers report from hot spot areas in Europe (14, 15) or regions of high prevalence in Germany (16). The extent, duration and the protective function of the antibody response are not clear. The infection rate in northern Germany has been milder than in other parts of the country. Due to rigorous containment measures and early contact tracing, the city of Luebeck had an incidence rate that was lower than the average incidence in Germany.

In the present study, we explored the dynamics of the antibody response with respect to onset, level and duration in patients with confirmed SARS-CoV-2 infection in this low incidence region. Most of the study patients were outpatients with either mild, moderate or even without symptoms. The disease severity of all patients was manageable by doctors in general practice (GP) or as outpatients in the local clinics. The precise knowledge of the disease severity allowed us to attempt the clinical validation of the antibody development.

MATERIALS AND METHODS

Study Population and Participant Recruitment

The city of Luebeck, with a population of 220,238 inhabitants, is situated in Northern Germany. The first two laboratory-confirmed SARS-CoV-2 cases were detected on February 29, 2020. The epidemic grew to 166 cases from which 151 recovered and one person (79 years) died (as of July 31, 2020) (**Figure 1 above**). A total of 166 confirmed COVID-19 patients

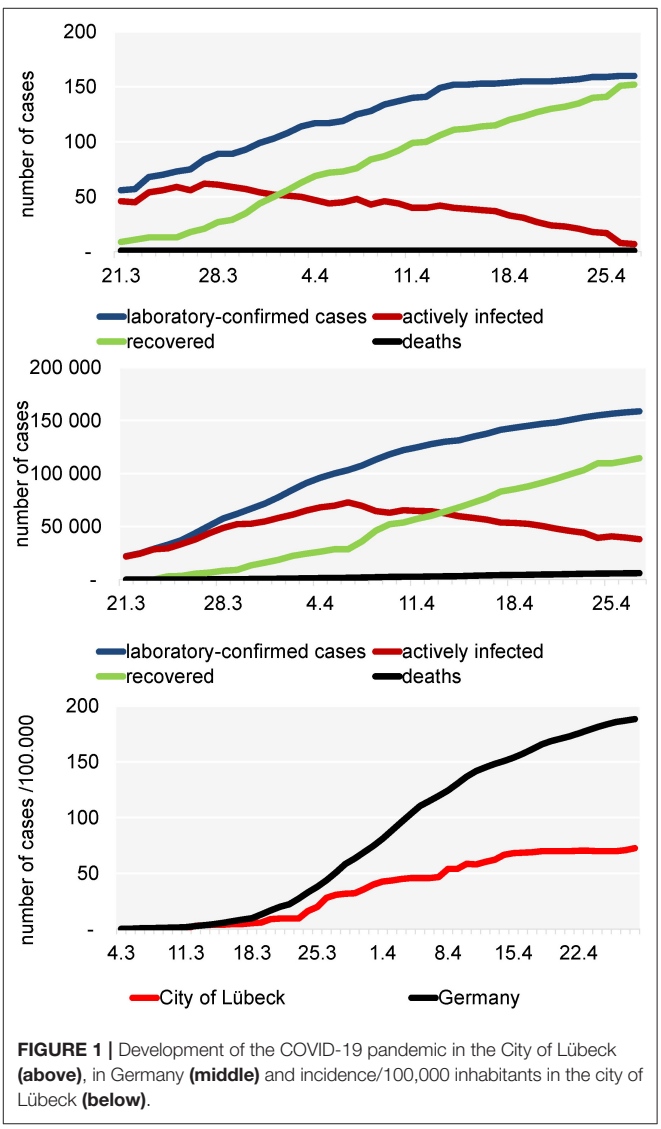


FIGURE 1 | Development of the COVID-19 pandemic in the City of Lübeck **(above)**, in Germany **(middle)** and incidence/100,000 inhabitants in the city of Lübeck **(below)**.

Abbreviations: COVID-19, coronavirus disease 19; ELISA, enzyme-linked immunosorbent assay; IgG/A, immunoglobulin G/A; rtPCR, real-time polymerase chain reaction; SARS-CoV-2, Severe acute respiratory syndrome coronavirus 2.

[1]https://www.rki.de/DE/Content/InfAZ/N/Neuartiges_Coronavirus/Situationsberichte/2020-03-26-de.pdf?__blob=publicationFile.

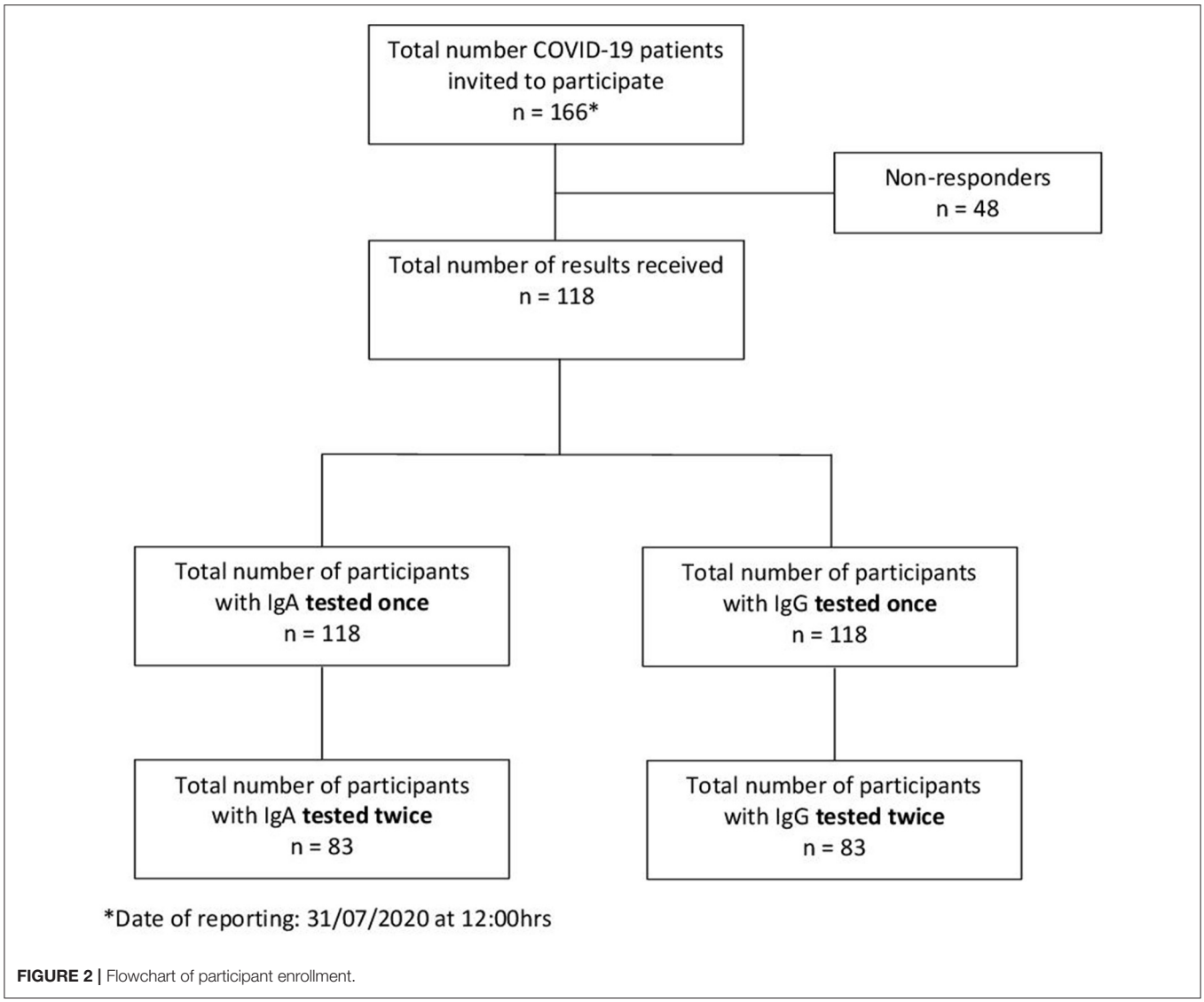


FIGURE 2 | Flowchart of participant enrollment.

who fulfilled the RKI definition[2] were detected by the local health authority between February 27, and July 31, 2020 (**Figure 2**) and were enrolled in the study. All enrolled cases were confirmed to be SARS-CoV-2 infected by use of a standard polymerase chain reaction (PCR) assay on throat swab samples from the respiratory tract taken by the local health authority. According to the guidelines, patients were quarantined routinely for at least 14 days from the onset of symptoms or from laboratory testing, respectively, in the absence of symptoms. Of these 166 index cases, 118 gave their written informed consent to participate (**Figure 2**). For children under the age of 18 years parents or other legal guardians provided "informed permission/consent" for study participation. For all of the enrolled patients, the date of symptom onset, disease severity (e.g., hospitalization) and demographic information were obtained from clinical records available to the local health authority and through a self-reported questionnaire.

Test Procedures

Detection of SARS-CoV-2

Nasopharyngeal swabs were taken from suspected COVID-19 cases by trained professionals either in a general practice (GP) or in a "drive-in" swab center run by the Health Protection Authority. Swabs were stored in stabilization media and processed immediately within 4 h, following DIN EN ISO 17025 und 15189 quality criteria, in the "Laboraerztliche Gemeinschaftspraxis Luebeck," which is located in the immediate vicinity of the "drive-in" swab center. SARS-CoV-2 RNA was detected qualitatively by using an automated one step real-time RT-PCR (RIDA®GENE SARS-CoV-2 RUO Test; R-Biopharm AG, Darmstadt, Germany; E-gene amplification) run on a RIDA®CYCLER according to the manufacturer's instruction.

[2]https://www.rki.de/DE/Content/InfAZ/N/Neuartiges_Coronavirus/Falldefinition.pdf?__blob=publicationFile

Detection of IgG and IgA Against SARS-CoV-2

Anti-SARS-CoV-2 IgG and IgA were detected by automated enzyme-linked immunosorbent assay - ELISA (product EI 2606-9601 G or A; EUROIMMUN; https://www.euroimmun.com) according to the manufacturer's instructions. The ELISA plates are coated with recombinant protein expressed S1 domain glycoprotein as antigen of the SARS-CoV-2. According to the latest data sheet (April 2020), the specificity for IgG testing is reported to be 99.1% for IgG and 88.5% for IgA, respectively. The data sheet reports cross-reactivities with SARS-CoV-1, but not with MERS-CoV, HCoV-229E, HCoV-NL63, HCoVHKU-1, or HCoV-OC43 virus. Thus, possible cross-reactivities are, at most, of marginal importance for this study, since very little, if any, SARS-CoV-1 infection is to be expected. The optical density (OD) was detected at 450 nm. A ratio of the OD of each sample to the reading of the calibrator, included in the kit, was automatically calculated according to the formula: OD ratio = OD of serum sample/OD of calibrator. According to the manufacturer, a ratio below 0.8 was evaluated as negative, 0.8 –<1.1 as borderline and >-1.1 as positive.

Statistical Analysis

Baseline and demographic characteristics of the patients were summarized by standard descriptive statistics. In order to investigate the seroconversion, the data from 118 sera samples were divided into six time windows of collection after symptom onset:

- 0–7 days
- 8–14 days
- 15–21 days
- 22–28 days
- 29–36 days
- 37 and more days

Categorical variables are expressed as numbers and percentages. Since data was not normally distributed, non-parametric tests were chosen. Spearman's correlation was conducted to assess the correlation between age and disease severity and antibody load (IgA and IgG load). A $p < 0.05$ was considered statistically significant. Statistical analyses were conducted using SPSS version 26.0.

RESULTS

Local and National Development of the COVID-19 Pandemic

Figure 1 above shows that from March 21 the number of active cases increased in a linear fashion and reached a plateau-like curve after April 16. From April 1 on, the number of recovered patients always exceeded the active cases. Until July 30, in total 166 COVID-19 laboratory-confirmed cases have been reported to the local health authority of Luebeck. One patient died due to COVID-19. For Germany, until April 30th, in total 161.539 COVID-19 laboratory-confirmed cases and 6.467 deaths due to COVID-19 were reported (**Figure 1 middle**). After March 16, the local incidence rate always was lower than the national incidence rate (**Figure 1 below**) and since April 15 it was relatively stable at 68.1–72.6/100,000, while the incidence in the rest of Germany was steadily increasing (∼189/100,000) as of April 27. A total of 118 patients with a confirmed diagnosis of COVID-19 were included in this study. All patients were positive for SARS-CoV-2 according to PCR testing of nasopharyngeal swabs.

TABLE 1 | Clinical and demographic characteristics of the patients included ($n = 118$).

Characteristics	*N* (%)
Gender	
Female	67 (56.8)
Male	51 (43.2)
Age groups	
10–19	7 (5.9)
20–29	22 (18.6)
30–39	18 (15.3)
40–49	14 (10.2)
50–59	34 (28.8)
60–69	17 (14.4)
70–79	6 (5.1)
80 and older	2 (1.7)
Disturbance of smell and/or taste (data available for 105 patients)	
Yes	64 (61.0)
No	41 (39.0)
Disease category (data available for 105 patients)	
1. No symptoms	6 (5.7)
2. Feeling of illness, but temperature < 38°C	45 (42.9)
3. General weakness, dry cough, temperature >38°C (influenza like illness)	45 (42.9)
4. As in 3 plus shortness of breath, signs of pneumonia	6 (5.7)
5. As in 4, hospital treatment required	3 (2.9)

Sample Characteristics

Clinical and demographic characteristics are reported in **Table 1**. Among all notified cases, 7 (5.9%) were children or adolescents aged 10–19 years, 88 (72.9%) persons were aged 20–59 years, 23 (19.5%) persons were aged 60–79 years, 2 (1.7%) persons were aged 80 and older (**Table 1**). Sixty-seven patients (56.8%) were female and 51 patients (43.2%) were male (**Table 1**).

Disturbance of Smell and Taste

More than half (61%) of the patients self-reported slight to massive decrease in one or both senses (i.e., taste or smell). Although we did not quantify, they reported a duration between one and up to 4 weeks after recovery from the acute illness.

Disease Severity

Patients exhibiting one or more of the following conditions were classified as having severe COVID-19: Fever above 38°C and signs of pneumonia accompanied by shortness of breath (i.e., category 4); Hospitalization (respiratory failure requiring mechanical ventilation and ICU care) (i.e., category 5).

Patients not meeting the above criteria, but exhibiting one or more of the following conditions classified as having mild COVID-19: feeling of illness, but temperature < 38°C (i.e., category 2); General weakness, dry cough, temperature >38°C (influenza like illness) (i.e., category 3).

Among the 118 patients, ∼6% of showed no symptoms; 86% had mild COVID (category 2 and 3) and 9% were severe cases (category 4 and 5) (**Table 1**). The main symptom was general weakness with or without headache or body ache, but no fever. We could not find a significant correlation with age and disease severity ($p > 0.05$) and neither an association between gender and disease severity ($p > 0.05$).

Antibody Profiling

As of July 31, 2020, IgA and IgG antibody results from 118 participants were available. A total of 83 patients were tested twice to monitor the course of antibody development (**Figure 2**).

Antibody levels for the first and second testing are shown in **Table 2**. According to the manufacturer's data sheet, an antibody ratio ≥ 1.1 is defined as positive, where as an antibody ratio < 0.8 is defined as negative. In the present sample, 21.7 and 18.1% of patients were seronegative for IgA or IgG, respectively (**Table 2**). Clinically, most of the seronegative patients showed no to only moderate symptoms. Among the six asymptomatic patients, two patients did not develop IgG antibodies (not shown). Twenty nine (24.5%) patients and 34 (28.8%) patients had high IgG and IgA antibody levels above 5 in the first testing, respectively (**Table 2**). Again, most of them had no to moderate symptoms thus were in category 1 to 3 (not shown).

Figures 3, **4** show the antibody levels for IgA and IgG, respectively, in relation to symptom onset. Antibodies were analyzed between day 7 and day 67 after symptom onset. As shown in **Figure 3** and **Table 3**, anti-SARS-CoV-2 S-specific IgA and IgG antibodies were not detectable in the very early days of infection (from day 0 to day 6). The first positive signals were detected at day 7 for IgA antibodies. Positive levels for IgG were detected between 8 and 14 days after symptom onset (**Figures 3**, **4**, **Table 3**). There was a wide inter-individual variation in the antibody levels. **Figure 5** indicates that IgA levels remain fairly stable, but increase at around day 50. A similar pattern could be detected for IgG levels, although IgA levels seem to increase over time (**Figure 6**).

TABLE 2 | IgA and IgG antibody levels against SARS-CoV-2 in COVID-19 patients.

Antibody levels	IgA (1)* ($n = 118$) *N* (%)	IgA (2)* ($n = 83$) *N* (%)	IgG (1)** ($n = 118$) *N* (%)	IgG (2)** ($n = 83$) *N* (%)
< 0.8	23 (19.5)	18 (21.7)	28 (23.7)	15 (18.1)
0.8–0.9	6 (5.1)	4 (4.8)	7 (5.9)	3 (3.6)
1.0–2.0	21 (17.8)	25 (30.1)	25 (21.2)	18 (21.7)
2.1–5.0	34 (28.8)	22 (26.5)	29 (24.6)	23 (27.7)
5.1–10.0	29 (24.6)	11 (13.3)	26 (22.0)	23 (27.7)
10.1–15.0	2 (1.7)	2 (2.4)	3 (2.6)	1 (1.2)
15.1–20.0	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
20.1–25.0	1 (0.8)	0 (0.0)	0 (0.0)	0 (0.0)
≥25.1	2 (1.7)	1 (1.2)	0 (0.0)	0 (0.0)

*(1) First test ** (2) Second test.

DISCUSSION

Our analysis of SARS-CoV-2 cases provides insight into the development of immune response in patients with light or moderate course of COVID-19 disease or asymptomatic patients.

Our cohort of patients was defined as having had viral mRNA in nasopharyngeal swabs by qualitative RT-PCR. The viral load was not analyzed quantitatively. The severity of diseases was very variable from completely asymptomatic to pneumonia-like symptoms. The severity of the symptoms may be dependent on

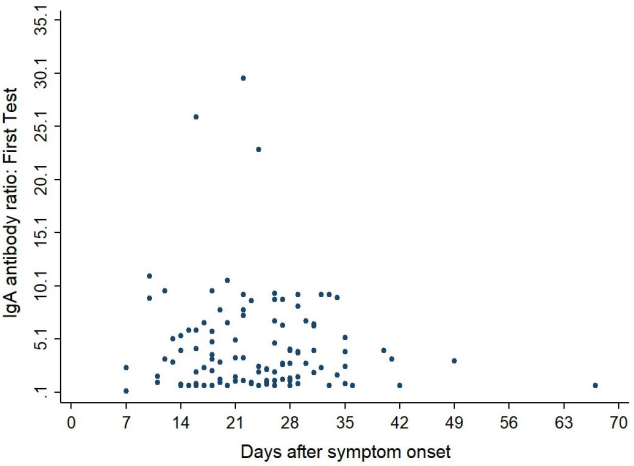


FIGURE 3 | Simple Scatter of IgA Antibodies by days after symptom onset.

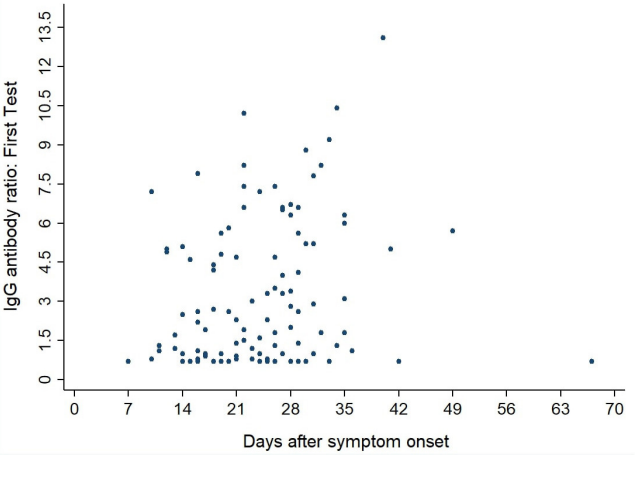


FIGURE 4 | Simple Scatter of IgG Antibodies by days after symptom onset.

the viral load at the time of infection. In Germany, the first and only commercially available test kit came into the market as of March 25, 2020. Based on the experience with the SARS-CoV-1 epidemics in 2003, the manufacturer (who also offers an antibody kit detecting SARS-CoV-1) decided to include IgA and IgG. The rationale behind this was, as suggested, that IgA would be much earlier and more specific than IgM and was expected to indicate mucosal immunity.

We analyzed IgA and IgG antibodies recognizing the S1 spike glycoprotein of SARS-CoV-2 in the serum of 118 SARS-CoV-2 PCR-positive patients with mild-to-moderate symptomatic or asymptomatic course in a low COVID-19-incidence region in northern Germany. Overall, more women (56.8%) than men tested positive for SARS-CoV-2, which is in line with overall findings for Germany as a whole (52%)[3].

With regard to the initial stratification the course of the disease was mild to moderate. Most patients, with a few exceptions, did not require hospital treatment, but most were treated by their general practitioner. Patients were treated by their family doctors. More than half self-reported sudden disturbances of smell and/or taste with varying durations (days to weeks) (17). The extent of olfactory dysfunction did not correlate with age, sex, or severity of the disease (not shown).

The antibody tests specifically interact with S1. It is likely, that other antigens like the viral nuclecapsid (NCP) antigen also induce antibody production. In the analysis of antibody levels, it appeared that, within a period of 50 days after the infection, 95/118 (81%) and 90/118 patients (76%) of the patients developed antibodies for IgA and IgG, respectively, above the threshold ratio of 1.1 at the first testing (**Table 2**). The level of antibodies, however, did not correlate significantly with age or sex or disease severity. Remarkably, 18% of the patients, with or without symptoms, did not develop IgG antibodies above the cut-off-value (1.1) after two testings. Again, no correlation with sex, age or disease severity could be observed (not shown). Our findings are in line with recent reports by colleagues who reported around 30% IgG-negative patients after SARS-CoV-2-infection (18). Others recently reported that mild disease – like in most of our cases – may stimulate mucosal SARS-CoV-2 secretion and IgG production may be associated mainly with severe cases (19).

The accumulating evidence supports a role for T cells in COVID-19 and probably in the immunological memory that forms following recovery from SARS-CoV-2 infection (20). Most, although not all, patients who are hospitalized seem to mount both CD8+ and CD4+ T cell responses, and evidence points to possible suboptimal (lymphopenia), excessive or otherwise inappropriate T cell responses associated with severe disease. Very rare data are available on T-cell responses from patients with mild to moderate disease like in our cohort.

It remains to be determined, whether S1 or NCP has the better positive or negative predictive value, as far as protection or resistance to re-infection is concerned.

[3]https://www.rki.de/DE/Content/InfAZ/N/Neuartiges_Coronavirus/Situationsberichte/2020-05-05-de.pdf?__blob=publicationFile

TABLE 3 | IgA and IgG detection in positive COVID-19 patients at different periods after disease onset.

Days after symptom onset	IgA positive* (ratio ≥1) *N* (%)	IgG positive* (ratio ≥1) *N* (%)
0–7	1 (1.1)	0 (0.0)
8–14	10 (11.2)	10 (12.0)
15–21	24 (27.0)	19 (22.9)
22–28	31 (34.8)	30 (36.1)
29–36	20 (22.5)	21 (25.3)
≥37	3 (3.4)	3 (3.7)

Antibody levels here coded as binary outcome: IgA level ≥1.0 = positive; IgA level <1.0 = negative; IgG level ≥1.0 = positive; IgG level <1.0 = negative.

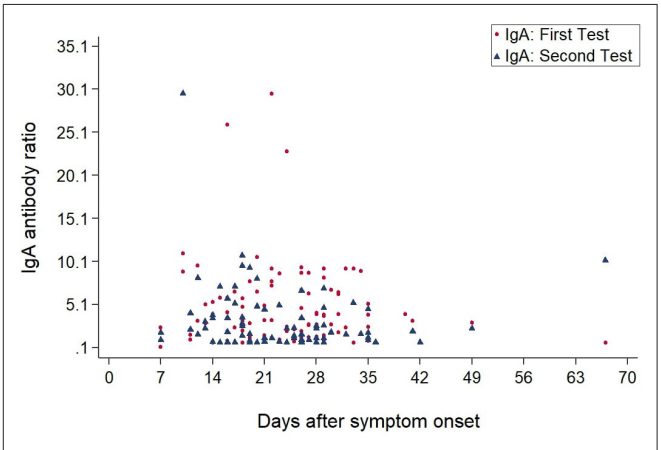


FIGURE 5 | IgA antibody development by days after symptom onset.

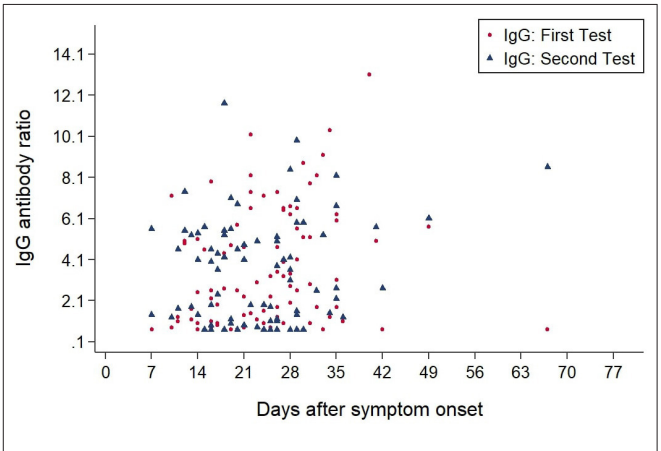


FIGURE 6 | IgG antibody development by days after symptom onset.

We can only speculate that the antibodies are neutralizing. Therefore, at this time, it will not be possible to testify protection for recovered patients. The severity of symptoms may be dissociated from the antigenicity or presentation of the S1 glycoprotein to the immune system. This assumption would

explain the high antibody variability which was not related to expression of clinical symptoms.

Six (5.7%) of the patients did not develop any SARS-CoV-2 specific symptoms. One out of these did not have detectable antibodies within 50 days. Thus, a negative antibody test does not rule out infection. The infectious inoculum possibly was not sufficiently high to induce disease and a subsequent immune reaction. Thus, both, a combination of SARS-CoV-2 PCR and a specific serological test are required to rule out infection (21).

One of the asymptomatic patients had an IgG ratio > 5. Although unlikely, it cannot be excluded entirely, that the patient was infected some weeks before the virus was detected and, thus, would have been protected in the observation period of this study. In any case, the data suggest that activation of the humoral immunity might require less virus than activation of symptomatic disease processes. Further studies are needed to define the respective minimal viral loads.

When we investigated the antibody levels in relation to time, it was obvious that there was a great diversity for both IgA and IgG. For IgA we found one positive value (IgA ratio ≥ 1.1) starting at day 7 after symptom onset (**Table 3**). For IgG, positive values (IgG ratio ≥ 1.1) were detected roughly 8–14 days after disease onset (**Table 3**). This is in line with other research that found that among most laboratory-confirmed COVID-19 cases, antibodies start to be detectable around 5–14 days after onset of symptoms (22–25). The test systems used were different. It remains to be seen, which antigen (nucleocapsid, spike glycoprotein) is the best to detect relevant antibodies. We could not find a correlation between antibody levels and age ($p > 0.05$).

Based on these findings, it is clear, that antibody diagnosis is a significant pillar to identify COVID-19 -positive patients in addition to SARS-CoV-2 PCR. It also will be indispensable for management of the pandemic. It is likely, that antibodies directed against the S1 glycoprotein are neutralizing (16).

In the cited study, most of the patients, but not all, with an IgG ratio above 2, had positive neutralization titres. They used the same test system as we did in our study. Thus, our data add information, but do not prove protection. For the individual patient, however, it cannot be answered at present, which antibody level will be protective and whether antibody-positive individuals are able to transmit the disease. Further studies are needed to answer this question, which is of utmost importance e.g., for health care workers.

There are some limitations in our study. Cross-reactivity could possibly be a limitation of immunoassays. On the one hand, our test was validated with a sensitivity of 89–100% and a specificity of 87.5–95.5% for IgA and 83.5–97.5% for IgG and a recent study has demonstrated negligible cross-reactivity from other human coronavirus NL63 to SARS-CoV-2 (12, 26). Our study does not give information on protective antibody functions with regard to resistance to re-infection and reduction of transmissibility of the virus. The results on the neutralization capacity are not present till now. Based on the development of IgG antibody dynamics, however, it might be reasonable to assume that ratios beyond 2 might confer protection. Nonetheless, this study provides valuable information regarding the seroconversion response, especially for IgA and IgG antibody development.

CONCLUSION

In the present study we detected that ∼2 weeks after infection the majority of symptomatic patients develop IgA and IgG antibodies. Our findings demonstrate that antibody tests have important diagnostic value in addition to RNA tests. In patients with viral RNA detection by PCR, but in the absence of symptoms, significant antibody levels were not detectable in a relevant proportion. This finding raises the question of false-positive PCR results that has to be investigated in further studies. Our data indicate, however, that antibody-positivity is a useful indicator of a previous SARS-CoV-2 infection. Negative antibodies cannot rule out SARS-CoV-2 infection. A number of questions still have to be answered. For the clinic, the determination of the neutralizing capacity of the antibodies in plasma therapy regimes will be of utmost relevance. On a population level, the protective effect for re-infections needs to be determined.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics committee University of Lübeck. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

WS and JS designed the study and analyzed the data. IB, DB, and TH performed statistical analysis and database management. RS delivered clinical information and serum specimens. BT and AB performed laboratory tests (PCR and antibody tests). WS, JS, and AM wrote the manuscript. All authors reviewed the manuscript and gave their consent.

ACKNOWLEDGMENTS

The authors would like to thank the patients sharing their data, all medical and laboratory personnel and administrative staff that contributed to obtain the results. This manuscript has been released as a pre-print at medRxiv (27).

REFERENCES

1. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet*. (2020) 395:497–506. doi: 10.1016/S0140-6736(20)30183-5
2. Zhu N, Zhang D, Wang W, Li X, Yang B, Song J, et al. A Novel Coronavirus from Patients with Pneumonia in China, 2019. *N Engl J Med*. (2020) 382:727–33. doi: 10.1056/NEJMoa2001017
3. Onder G, Rezza G, Brusaferro S. Case-fatality rate and characteristics of patients dying in relation to COVID-19 in Italy. *JAMA*. (2020). doi: 10.1001/jama.2020.4683. [Epub ahead of print].
4. Bi Q, Wu Y, Mei S, Ye C, Zou X, Zhang Z, et al. Epidemiology and transmission of COVID-19 in 391 cases and 1286 of their close contacts in Shenzhen, China: a retrospective cohort study. *Lancet Infect Dis*. (2020) 20:911–9. doi: 10.1016/S1473-3099(20)30287-5
5. Gudbjartsson DF, Helgason A, Jonsson H, Magnusson OT, Melsted P, Norddahl GL, et al. Spread of SARS-CoV-2 in the Icelandic Population. *N Engl J Med*. (2020) 382:2302–15. doi: 10.1056/NEJMoa2006100
6. He X, Lau EHY, Wu P, Deng X, Wang J, Hao X, et al. Temporal dynamics in viral shedding and transmissibility of COVID-19. *Nat Med*. (2020) 26:672–5. doi: 10.1038/s41591-020-0869-5
7. Hijnen D, Marzano AV, Eyerich K, GeurtsvanKessel C, Gimenez-Arnau AM, Joly P, et al. SARS-CoV-2 transmission from presymptomatic meeting attendee, Germany. *Emerg Infect Dis*. (2020) 26:1935–7. doi: 10.3201/eid2608.201235
8. Bohmer MM, Buchholz U, Corman VM, Hoch M, Katz K, Marosevic DV, et al. Investigation of a COVID-19 outbreak in Germany resulting from a single travel-associated primary case: a case series. *Lancet Infect Dis*. (2020) 20:920–8. doi: 10.1016/S1473-3099(20)30314-5
9. Rothe C, Schunk M, Sothmann P, Bretzel G, Froeschl G, Wallrauch C, et al. Transmission of 2019-nCoV infection from an asymptomatic contact in Germany. *N Engl J Med*. (2020) 382:970–1. doi: 10.1056/NEJMc2001468
10. Lipsitch M, Swerdlow DL, Finelli L. Defining the epidemiology of Covid-19 - studies needed. *N Engl J Med*. (2020) 382:1194–6. doi: 10.1056/NEJMp2002125
11. Chan JF, Yip CC, To KK, Tang TH, Wong SC, Leung KH, et al. Improved molecular diagnosis of COVID-19 by the novel, highly sensitive and specific COVID-19-RdRp/hel real-time reverse transcription-PCR assay validated *in vitro* and with clinical specimens. *J Clin Microbiol*. (2020) 58:e00310-20. doi: 10.1128/JCM.00310-20
12. Okba NMA, Muller MA, Li W, Wang C, GeurtsvanKessel CH, Corman VM, et al. Severe acute respiratory syndrome coronavirus 2-specific antibody responses in coronavirus disease patients. *Emerg Infect Dis*. (2020) 26:1478–88. doi: 10.3201/eid2607.200841
13. Wolfel R, Corman VM, Guggemos W, Seilmaier M, Zange S, Muller MA, et al. Virological assessment of hospitalized patients with COVID-2019. *Nature*. (2020) 581:465–9. doi: 10.1038/s41586-020-2196-x
14. Dimeglio C, Loubes JM, Deporte B, Dubois M, Latour J, Mansuy JM, et al. The SARS-CoV-2 seroprevalence is the key factor for deconfinement in France. *J Infect*. (2020) 81:318–56. doi: 10.1016/j.jinf.2020.04.031
15. Pollan M, Perez-Gomez B, Pastor-Barriuso R, Oteo J, Hernan MA, Perez-Olmeda M, et al. Prevalence of SARS-CoV-2 in Spain (ENE-COVID): a nationwide, population-based seroepidemiological study. *Lancet*. (2020) 396:535–44. doi: 10.1016/S0140-6736(20)31483-5
16. Streeck H, Schulte B, Kuemmerer BM, Richter E, Hoeller T, Fuhrmann C. Infection fatality rate of SARS-CoV-2 infection in a German community with a super-spreading event. *Preprint*. (2020). doi: 10.1101/2020.05.04.20090076
17. De Maria A, Varese P, Dentone C, Barisione E, Bassetti M. High prevalence of olfactory and taste disorder during SARS-CoV-2 infection in outpatients. *J Med Virol*. (2020). doi: 10.1002/jmv.25995. [Epub ahead of print].
18. Robbiani DF, Gaebler C, Muecksch F, Lorenzi JCC, Wang Z, Cho A, et al. Convergent antibody responses to SARS-CoV-2 in convalescent individuals. *Nature*. (2020) 584:437–42. doi: 10.1038/s41586-020-2456-9
19. Cervia C, Nilsson J, Zurbuchen Y, Valaperti A, Schreiner J, Wolfensberger, A et al. Systemic and mucosal antibody secretion specific to SARS-CoV-2 during mild versus severe COVID-19. *Preprint*. (2020). doi: 10.1101/2020.05.21.108308
20. Chen Z, John Wherry E. T cell responses in patients with COVID-19. Nat Rev Immunol. (2020) 20:529–36. doi: 10.1038/s41577-020-0402-6
21. Wu X, Fu B, Chen L, Feng Y. Serological tests facilitate identification of asymptomatic SARS-CoV-2 infection in Wuhan, China. *J Med Virol*. (2020). doi: 10.1002/jmv.25904. [Epub ahead of print].
22. Peeling RW, Wedderburn CJ, Garcia PJ, Boeras D, Fongwen N, Nkengasong J, et al. Serology testing in the COVID-19 pandemic response. *Lancet Infect Dis*. (2020) 20:e245–49. doi: 10.1016/S1473-3099(20)30517-X
23. To KK, Tsang OT, Leung WS, Tam AR, Wu TC, Lung DC, et al. Temporal profiles of viral load in posterior oropharyngeal saliva samples and serum antibody responses during infection by SARS-CoV-2: an observational cohort study. *Lancet Infect Dis*. (2020) 20:565–74. doi: 10.1016/S1473-3099(20)30196-1
24. Xiang F, Wang X, He X, Peng Z, Yang B, Zhang J, et al. Antibody detection and dynamic characteristics in patients with COVID-19. *Clin Infect Dis*. (2020). doi: 10.1093/cid/ciaa461. [Epub ahead of print].
25. Zhao J, Yuan Q, Wang H, Liu W, Liao X, Su Y, et al. (2020). Antibody responses to SARS-CoV-2 in patients of novel coronavirus disease 2019. *Clin Infect Dis*. (2020). doi: 10.1093/cid/ciaa344. [Epub ahead of print].
26. Amanat F, Stadlbauer D, Strohmeier S, Nguyen THO, Chromikova V, McMahon M, et al. A serological assay to detect SARS-CoV-2 seroconversion in humans. *Nat Med*. (2020) 26:1033–6. doi: 10.1038/s41591-020-0913-5
27. Solbach W, Schiffner J, Backhaus I, Burger D, Staiger R, Tiemer B, et al. Antibody profiling of COVID-19 patients in an urban low-incidence region in Northern Germany. *Preprint*. (2020). doi: 10.1101/2020.05.30.20111393

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Public Health

BRIEF RESEARCH REPORT
published: 22 September 2020
doi: 10.3389/fpubh.2020.550933

The CHIR Score for Evaluating the Hyperimmune Response in COVID-19: A Preliminary Concept

Daniel Kumar Goyal[1,2]* *and Fatma Mansab*[2,3]

[1] Gibraltar COVID-19 Research Group, University of Gibraltar, Gibraltar, United Kingdom, [2] Acute General Medicine, Department of Medicine, St Bernard's Hospital, Gibraltar Health Authority, Gibraltar, United Kingdom, [3] Health Advisory Unit, Public Health Gibraltar, Gibraltar, United Kingdom

Similar to SARS and MERS, the host immune response to COVID-19 is implicated in the severity of the disease itself. Here, we investigate the possible use of scoring systems to help guide clinicians in their determination as to when to commence immunosuppressive treatment in COVID-19. We utilized the relatively established clinical and biochemical severity indicators from large cohort studies to develop a potential scoring system for the hyperimmune response in COVID-19.

Keywords: COVID-19, SARS-CoV-2, 2019-nCov, hyperimmune, cytokine storm, corticosteroids, treatment, inflammation

OPEN ACCESS

Edited by:
Zisis Kozlakidis, International Agency for Research on Cancer (IARC), France

Reviewed by:
James Robert Neil, Centre for Nutrition Education and Lifestyle Management (CNELM), United Kingdom
Gaurav Chauhan, University of Massachusetts Medical School, United States

***Correspondence:**
Daniel Kumar Goyal
daniel.goyal@unigib.edu.gi

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Public Health

Received: *10 April 2020*
Accepted: *26 August 2020*
Published: *22 September 2020*

Citation:
Goyal DK and Mansab F (2020) The CHIR Score for Evaluating the Hyperimmune Response in COVID-19: A Preliminary Concept. Front. Public Health 8:550933. doi: 10.3389/fpubh.2020.550933

INTRODUCTION

SARS-CoV2 causes COVID-19 (Coronavirus Disease 2019). As of April 2020, the total reported cases of COVID-19 was over 1.5 million, with 100,000 deaths over 4 months. More than half these deaths have occurred in the last month.

Severity of COVID-19 can be associated with prolonged fever, rising inflammatory markers and signs of systemic inflammation such as bone marrow suppression, in the absence of secondary infection (1). A growing body of evidence suggests such an inflammatory response may be more related to a hyperimmune response as opposed to the direct effects of the virus.

Total viral load in COVID-19 seems to decrease after the initial phase of the infection (2), and early evidence suggests such a reduction in viral load does not correlate with severity or mortality outcomes (3, 4), although there is a report of correlation between stage of disease (early, progressive, and recovery) and level of virus in nasopharyngeal swabs (5). It seems individuals with low viral loads can still progress to severe lung pathology, and equally, those with similar viral loads can suffer only mild symptoms and recover quickly (5).

Postmortem examinations have identified marked inflammatory changes within the lung tissue, together with virally activated T-cells without intranuclear or intracytoplasmic viral inclusions. This led investigators to suggest that based on the pathological findings in lung tissue, immunosuppressive medication would be indicated (6).

Such a hyperimmune response can be characterized in a number of ways. Cytokine Release Syndrome (CRS), Haemophagocytic Syndrome (HS), and Cytokine Storm Syndrome (CSS) have all been described in COVID-19 (7, 8). Commonalities between these different hyperimmune responses include, systemic upset with pyrexia and malaise; rising inflammatory markers with bone marrow suppression, and eventually Systemic Inflammatory Response Syndrome (SIRS) and Acute Respiratory Distress Syndrome (ARDS).

Similar findings were discovered in SARS and MERS (9, 10).

Most of these hyperimmune states respond to disease modifying agents. For example, Cytokine Release Syndrome is often responsive to Tocilizumab or corticosteroids (11). Haemophagocytic Syndrome is managed with corticosteroids, intravenous Immunoglobulin or Tocilizumab (8).

Disease Modifying Agents in SARS-Cov2

SARS treatment was developed during the outbreak itself, based on clinical findings and experience. Over time, the mainstay of treatments included ribavarin, broad-spectrum antibiotics and corticosteroids. Such practice became established. This led to challenges in undertaking Randomized Contrlled Trials (RCT). As such, the evidence for these established interventions lacks the power to provide the certainty necessary to generate national or international guidelines (12). Still to this day, many different groups maintain different protocols for the treatment of SARS, although corticosteroids remain a cornerstone of intervention.

There is limited data on corticosteroid use in SARS-CoV2. One of the few studies report on a retrospective analysis of low-dose, short-term corticosteroid use in patients with severe COVID-19 infection. They report significant improvement in oxygenation and resolution of CT changes with 5–7 days of 1–2 mgs/kg/day of methylprednisolone vs. no methylprednisolone (8.2 days [IQR 7.0–10.3] vs. 13.5 days [IQR 10.3–16]; $P < 0.001$). There were only three deaths in the total cohort of 46, so no inference relating to mortality can be drawn (13).

A further trial is underway in China with a dosing schedule of 1–2 mgs/kg methylprednisolone for a duration of 3 days (14).

There are additional trials currently underway examining other disease modifying agents in COVID-19, including Tocilizumab, Immunoglobulin, and Convalescent Plasma.

GENERATING A HYPERIMMUNE SCORING SYSTEM

In this present study we aimed to generate an initial, testable hyperimmune score specific to COVID-19. Building on the suggestions of Cron et al. (7) and Mehta et al. (8), and with the increasing body of evidence supporting various inflammatory markers as disease severity indicators (15–17), we first took the validated hyperimmune score associate with hyperphagocytic syndrome, the HScore, and adapted it to COVID-19 specific clinical features (**Table 1**).

COVID-19 pneumonia typically presents with high CRP, relatively low PCT and often low lymphocytes (16, 17). The persistence of fever, further bone marrow suppression (e.g., thrombocytopenia)—in the absence of evidence for secondary infection—would be typically interpreted as a systemic inflammatory response. The COVID-19 Hyperimmune Score (CHIR Score) was designed to reflect these relative consistencies in clinical and pathological parameters. Crucially, the scoring system was designed to aid in the *confirmation* of a hyperimmune response, and *not* to have any negative predictive value (i.e., the CHIR Score cannot provide any guidance as to the absence of a hyperimmune response). As such scores were divided into Likely (Score 150 and above) and Possible (Score 80–150).

TABLE 1 | COVID-19 Hyperimmune Response (CHIR) scoring criteria.

Measure	Points
Temperature (C)	
38.4–39.4	30
>39.4	50
Days of symptoms	
3–7	30
7–10	15
White cell count (10^9/L)	
<6.0	30
Lymphocytes (10^9/L)	
<1.0	15
<0.5	30
AST (IU/L)	
>30	15
Platelets (10^9/L)	
<110	15
<90	30
<60	50
CRP (mg/L)	
>100	15
>200	30
Procalcitonin (ng/ml)	
<0.21	15
>0.5 and <0.8	−25
0.8 to 1.0	−50
>1.0	−75
Total score	**Hyperimmune Response**
<80	Unknown
80–149	Possible
>149	Likely

WCC, White Cell Count; CRP, C-Reactive Protein; PCT, Procalcitonin; AST, Aspartate Aminotransferase.

In determining the weighting for each clinical or pathological parameter we considered point measures with ease of repeating.

Fever was given a relatively substantive weighting. Whilst a hyperinflammatory syndrome can occur without fever (particularly in the elderly), the presence of fever is the most common symptom (11). There is limited evidence for the relationship between level of fever and severity of hyperimmune response, however the severity of fever is generally viewed as a marker of severity. As such, the CHIR score attributes a greater value to temperatures over 39.4°C.

The "Days to Onset" may not determine the probability of a hyperimmune response, but those who develop marked inflammatory changes early in the disease (in the absence of secondary infection) seem to be at risk of a more severe inflammatory cascade, consistent with other hyperinflammatory syndromes (7). As such the CHIR score attributes a higher score to a shorter history.

Abbreviations: SARS, Severe Acute Respiratory Syndrome; COVID-19, Coronavirus Disease 2019; SARS-CoV2, Severe acute respiratory syndrome coronavirus 2; MERS, Middle East Respiratory Syndrome.

White cell count, lymphocyte count and platelet count are attributed a positive score at varying levels in the CHIR score due to the frequent occurrence of bone marrow suppression in an acute hyperinflammatory syndrome (11, 18), and the quite consistent relationship between level of lymphopenia with severity (16, 17).

C-Reactive Protein (CRP) is a marker of inflammation, and as such has relevance in a hyperinflammatory condition. As it is a non-specific marker of inflammation—rising in infection, a hyperimmune response and malignancy -, it is not discriminatory between infection and inflammation, and as such is attributed a modest predictive score.

Evidence suggests procalcitonin (PCT) has discriminatory value between infective inflammation and non-infective inflammation (19). A significantly raised PCT is highly suggestive of bacterial infection. Given the main differential when considering a hyperinflammatory state is a secondary bacterial infection, PCT has added value, and has been attributed a high negative CHIR score.

The CHIR Score is only intended to be utilized where there is confidence that a secondary infection has been excluded. Patients with, for example, clear and confirmed immunosuppression (e.g., neutropenia) may well-achieve higher CHIR scores, but may be more likely to have superadded infection vs. a hyperimmune response. The negative scoring of significantly raised PCT serves to mitigate such patient groups, however clinical acumen remains a necessity in interpretation.

DISCUSSION

An international collaboration led by the American Thoracic Society recently issued emergency guidance on treatment recommendations in COVID-19. This pragmatic stance reflects the reality of the significant time-lag to the results of sufficiently powered RCTs, and the need for treatment options during the pandemic (20).

Physicians on the ground facing this new disease must make the best decisions they can based on their knowledge, experience, and the limited available data. Hyperimmune scoring systems such as the CHIR Score presented here may provide some support in the consideration of when to commence disease modifying agents or immunosuppressives such as corticosteroids in patients with severe COVID-19. As such, this publication builds on the suggestion made by Zhou et al. (21): tailored and responsive corticosteroids may well-offer survival benefit in SARS-CoV2.

The CHIR Score remains a preliminary concept. Future studies will include a retrospective analysis of the predictive power of the CHIR Score in determining treatment responsiveness in clinical trials involving immunosuppressive medications. If predictive, a prospective clinical trial would be required to validate the score as a usable clinical tool.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7143164/bin/NEJMoa2004500_appendix.pdf.

AUTHOR CONTRIBUTIONS

All authors contributed to the concept, design, write-up, and final approval of the article.

REFERENCES

1. Bhatraju PK, Ghassemieh BJ, Nichols M, Kim R, Jerome KR, Nalla AK, et al. Covid-19 in critically ill patients in the seattle region—case series. *N Engl J Med.* (2020) NEJMoa2004500. 382:2012–22. doi: 10.1056/NEJMoa2004500
2. Xu T, Chen C, Zhu Z, Cui M, Chen C, Dai H, et al. Clinical features and dynamics of viral load in imported and non-imported patients with COVID-19. *Int J Infect Dis.* (2020) 94:68–71. doi: 10.1016/j.ijid.2020.03.022
3. Zou L, Ruan F, Huang M, Liang L, Huang H, Hong Z, et al. SARS-CoV-2 viral load in upper respiratory specimens of infected patients. *N Engl J Med.* (2020) 382:1177–9. doi: 10.1056/NEJMc2001737
4. Lescure F-X, Bouadma L, Nguyen D, Parisey M, Wicky P-H, Behillil S, et al. Clinical and virological data of the first cases of COVID-19 in Europe: a case series. *Lancet Infect Dis.* (2020) 20:697–706. doi: 10.1016/S1473-3099(20)30200-0
5. Yu F, Yan L, Wang N, Yang S, Wang L, Tang Y, et al. Quantitative detection and viral load analysis of SARS-CoV-2 in infected patients. *Clin Infect Dis.* 71:793–8. doi: 10.1093/cid/ciaa345
6. Xu Z, Shi L, Wang Y, Zhang J, Huang L, Zhang C, et al. Pathological findings of COVID-19 associated with acute respiratory distress syndrome. *Lancet Respir Med.* (2020) 8:420–22. doi: 10.1016/S2213-2600(20)30076-X
7. Cron RQ, Chatham WW. The rheumatologist's role in covid-19. *J Rheumatol.* (2020) 47:639–42. doi: 10.3899/jrheum.200744
8. Mehta P, McAuley DF, Brown M, Sanchez E, Tattersall RS, Manson JJ, et al. COVID-19: consider cytokine storm syndromes and immunosuppression. *Lancet.* (2020) 395:1033–4. doi: 10.1016/S0140-6736(20)30628-0
9. Ding Y, Wang H, Shen H, Li Z, Geng J, Han H, et al. The clinical pathology of severe acute respiratory syndrome (SARS): a report from China. *J Pathol.* (2003) 200:282–9. doi: 10.1002/path.1440
10. Liu J, Zheng X, Tong Q, Li W, Wang B, Sutter K, et al. Overlapping and discrete aspects of the pathology and pathogenesis of the emerging human pathogenic coronaviruses SARS-CoV, MERS-CoV, and 2019-nCoV. *J Med Virol.* (2020) 92:491–4. doi: 10.1002/jmv.25709
11. Lee DW, Gardner R, Porter DL, Louis CU, Ahmed N, Jensen M, et al. Current concepts in the diagnosis and management of cytokine release syndrome. *Blood.* (2014) 124:188–95. doi: 10.1182/blood-2014-05-552729
12. Stockman LJ, Bellamy R, Garner P. SARS: systematic review of treatment effects. *PLoS Med.* (2006) 3:e343. doi: 10.1371/journal.pmed.0030343
13. Wang Y, Jiang W, He Q, Wang C, Wang B, Zhou, et al. Early, low-dose and short-term application of corticosteroid treatment in patients with severe COVID-19 pneumonia: single-center experience from Wuhan, China. *medRxiv*. medRxiv:2020.03.06.20032342. doi: 10.1101/2020.03.06.20032342
14. Qin Y-Y, Zhou Y-H, Lu Y-Q, Sun F, Yang S, Harypursat V, et al. Effectiveness of glucocorticoid therapy in patients with severe novel coronavirus pneumonia: protocol of a randomized controlled trial. *Chin Med J.* (2020) 133:1080–6. doi: 10.1097/CM9.0000000000000791
15. Carr E, Bendayan R, Bean D, Stammers M, Wang W, Zhang H, et al. Supplementing the National Early Warning Score (NEWS2) for anticipating early deterioration among patients with COVID-19 infection. *medRxiv*. doi: 10.1101/2020.04.24.20078006

16. Guan WJ, Ni ZY, Hu Y, Liang W-H, Ou C-Q, He J-X, et al. Clinical characteristics of coronavirus disease 2019 in China. *N Engl J Med*. (2020) 382:1708–20. doi: 10.1056/NEJMoa2002032
17. Richardson S, Hirsch JS, Narasimhan M, Crawford JM, McGinn T, Davidson KW, et al. Presenting characteristics, comorbidities, and outcomes among 5,700 patients hospitalized with COVID-19 in the New York City area. *JAMA*. (2020) 323:2052–9. doi: 10.1001/jama.2020.6775
18. Fardet L, Galicier L, Lambotte O, Marzac C, Aumont C, Chahwan D, et al. Development and validation of the HScore, a score for the diagnosis of reactive hemophagocytic syndrome. *Arthritis Rheumatol*. (2014) 66:2613–20. doi: 10.1002/art.38690
19. Covington EW, Roberts MZ, Dong J. Procalcitonin monitoring as a guide for antimicrobial therapy: a review of current literature. *Pharmacotherapy*. (2018) 38:569–81. doi: 10.1002/phar.2112
20. Stawicki SP, Jeanmonod R, Miller AC, et al. The 2019-2020 novel coronavirus (severe acute respiratory syndrome coronavirus 2) pandemic: a Joint American College of Academic International Medicine-World Academic Council of Emergency Medicine Multidisciplinary COVID-19 working group consensus paper. *J Glob Infect Dis*. (2020) 12:47–93. doi: 10.4103/jgid.jgid_86_20
21. Zhou W, Liu Y, Tian D, Wang C, Wang S, Cheng J, et al. Potential benefits of precise corticosteroids therapy for severe 2019-nCoV pneumonia. *Signal Transduct Target Ther*. (2020) 5:18. doi: 10.1038/s41392-020-0127-9

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Public Health

BRIEF RESEARCH REPORT
published: 22 September 2020
doi: 10.3389/fpubh.2020.562615

Geographical Distribution of Genetic Variants and Lineages of SARS-CoV-2 in Chile

*Andrés E. Castillo[1], Bárbara Parra[1], Paz Tapia[1], Jaime Lagos[1], Loredana Arata[1], Alejandra Acevedo[2], Winston Andrade[2], Gabriel Leal[2], Carolina Tambley[2], Patricia Bustos[2], Rodrigo Fasce[2] and Jorge Fernández[1]**

[1] Molecular Genetics Sub Department, Institute of Public Health of Chile, Santiago, Chile, [2] Section of Respiratory and Exanthematic Viruses, Institute of Public Health of Chile, Santiago, Chile

The pandemic caused by the new severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a worldwide public health concern. First confined in China and then disseminated widely across Europe and America, SARS-CoV-2 has impacted and moved the scientific community around the world to working in a fast and coordinated way to collect all possible information about this virus and generate new strategies and protocols to try to stop the infection. During March 2020, more than 16,000 full viral genomes have been shared in public databases that allow the construction of genetic landscapes for tracking and monitoring the viral advances over time and study the genomic variations present in geographic regions. In this work, we present the occurrence of genetic variants and lineages of SARS-CoV-2 in Chile during March to April 2020. Complete genome analysis of 141 viral samples from different regions of Chile revealed a predominance of variant D614G like in Europe and the USA and the major presence of lineage B.1. These findings could help take control measures due to the similarity of the viral variants present in Chile, compared with other countries, and monitor the dynamic change of virus variants in the country.

Keywords: COVID-19, SARS-CoV- 2, epidemiology, variants, lineages

OPEN ACCESS

Edited by:
Zisis Kozlakidis, International Agency For Research On Cancer (IARC), France

Reviewed by:
Fouzia Sadiq, Shifa Tameer-e-Millat University, Pakistan
Xianding Deng, University of California, San Francisco, United States

***Correspondence:**
Jorge Fernández
jfernand@ispch.cl

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Public Health

Received: *15 May 2020*
Accepted: *13 August 2020*
Published: *22 September 2020*

Citation:
Castillo AE, Parra B, Tapia P, Lagos J, Arata L, Acevedo A, Andrade W, Leal G, Tambley C, Bustos P, Fasce R and Fernández J (2020) Geographical Distribution of Genetic Variants and Lineages of SARS-CoV-2 in Chile. Front. Public Health 8:562615. doi: 10.3389/fpubh.2020.562615

INTRODUCTION

The rapid infection and spread of the new severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) made it necessary to adopt extraordinary measures, such as quarantines, social distancing, extreme hygienic procedures, and official useful information. In this line, the rapid sharing of trustable information plays a key role in providing guidance to physicians and investigators who advise the authorities to take the best decisions during each stage of the pandemic. Free and quick access to the latest scientific findings contributes to the management and control all aspects of the pandemic, be it scientific or social, without neglecting the quality of this information (1–3).

The outbreak of SARS-CoV-2 was declared as a pandemic on March 11, 2020 by the World Health Organization due to the rapid increase in the number of infected patients outside China (13-fold) and the growing number of countries (up to 113) with cases of coronavirus disease (COVID-19) (4). By August 12, 2020, there are more than 20,162,474 positive cases in 215 countries and territories, with a death toll of 737,417 worldwide (5).

Since the COVID-19 outbreak in China in later December 2019, almost 5 months later and with an unprecedent speed, investigators around the world have uploaded and shared near to 16,000 high coverage genomes, contributing to the development of new diagnostic strategies, tracking the strains for a better understanding of virus spread dynamics and vaccine and treatment development, among other valuable knowledge contributions.

Those genome sequences are hosted in the GISAID Initiative (https://gisaid.org/CoV2020), created to collect influenza viruses information, previous to the SARS-CoV-2 pandemic. To date, there is no official system for naming the phylogenetic diversity, making it confusing and difficult to reach a consensus about strains classification, but there are two ways to group the fast-growing number of isolates, in variants and lineages. According to the guidelines of the GISAID database, the genetic diversity of the isolates was categorized in clades as a consequence of specific single nucleotide polymorphism (SNP) present in the genome. The genetic variants are located in the nucleotide positions 23,403, 26,144, and 28,144 based on the reference sequence NC_045512.2, and the variant's name is represented by a capital letter that corresponds to the amino acid substitution product of the SNP G: Spike—D614G, V: NS3—G251V, S: ORF8—L84S, respectively, and O for other strains that keep some of the nucleotide as the reference strain on that genome position that cannot be assigned to the previous described clades (6). Tracking the cumulative SNPs along the genome has been used to identify the lineages related to the viral spread (7).

RNA viruses are ever-evolving structures, adapting constantly, due to the exposure to variable environments, and the lower viral fitness in these scenarios, for example, interspecies jumps and geographical dissemination, and the high error rates of the RNA-dependent RNA polymerases (RdRp) contribute to fit in the new ambient in just a few generations. The error rates in viral RNA polymerases are near to 10^{-4} compared with 10^{-7}-10^{-11} in DNA viruses (8, 9). However, SARS-CoV-2 possesses a non-structural gene with proof-reading activity; thus, its mutation rate is slow, at the pace of 1–2 base substitutions per month across the genome (10).

Up to date, several SNPs across the SARS-CoV-2 genome have been identified (11) in the genes involved in the life cycle of the virus and potential target for antivirals, such as RdRp (12) and Spike protein (13, 14). Until now, there is a few evidence to assign pathogenic or infective special features to the genomic variants (15), but we know that G variant is currently prevalent in the world, with 64% of the sequences found mainly in Europe and North America.

We have already published the phylogenetic analysis of the first four genomes detected in Chile that revealed the two variants derived from strains present mainly in Europe (16).

According to the last report of the Chilean Ministry of Health, until August 9, the cumulative COVID-19 cases had reached 418,196 patients, with 10,402 deaths, where the Metropolitan Region of Santiago concentrates the highest number of affected people, with 69.8% of infected patients and 79% of deaths in the country (17).

In this report, we show the geographical distribution of 141 SARS-CoV-2 isolates collected along the Chilean territory. The complete genome analysis of those samples allows us to identify and classify both genomic variants and lineages.

MATERIALS AND METHODS

Sample Types, RNA Extraction, and Virus Detection

Chilean law by the Supreme Decree 7/2019 mandates the notification of communicable diseases and their surveillance. Throat and nasopharyngeal swab samples were mainly collected. A volume of 140 μl of each sample was used for viral RNA extraction with QIAamp Viral Mini Kit (Qiagen, Cat. No. 52926) in a QIACube extractor. All suspicious cases were confirmed by real-time reverse transcription (RT)-PCR using TaqMan™ 2019-nCoV Assay Kit v1 (Thermo Fisher, Cat. No. A47532).

Full Viral Genome Amplification

Genome amplification must be performed in two-steps RT and conventional PCR in order to generate a total of 12 fragments around 2.3–2.7 Kbp (16). From total RNA extraction, we performed a first amplification round in order to obtain six cDNA fragments. Each fragment around 5 Kbp was amplified by RT-PCR using 5 μl of RNA, 400 nM of each primer, and the SuperScript® III one-step RT-PCR System with Platinum® Taq Kit (12.5 μl of reaction mix and 0.5 μl of RT/Taq mix, Invitrogen) in a 25 μl final volume. The thermal profile used was 60 min at 45°C, 2 min at 94°C, 40 cycles consisting of denaturation at 94°C, 15 s; annealing at 47°C, 30 s; and elongation at 68°C, 6 min, followed by a final extension for 5 min at 68°C. Each DNA product obtained in the first RT-PCR round is the substrate for the second PCR round, generating two fragments from each first round DNA product. PCR conditions using SapphireAmp fast PCR—hot-start master mix (Takara Bio USA, Cat. No. RR350B) were: initial denaturation for 2 min at 94°C, 30 cycles consisting of denaturation at 94°C, 30 s; annealing at 47°C, 30 s; elongation at 72°C, 1 min; and final extension at 72°C, 5 min.

Library Generation and Sequencing

The 12 DNA fragments from full genome amplification were pooled, and libraries were prepared with the Nextera XT Library Prep Kit (Illumina, San Diego, CA, USA), purified with Agencourt AMPure XP beads (Beckman Coulter, Brea, CA, USA), and quantified by Victor Nivo Fluorometer (PerkinElmer) using Quant-it dsDNA HS Assay Kit (Invitrogen). The resulting DNA libraries were sequenced on MiSeq (Illumina) using a 300-cycle (total) reagent kit. About 0.3 GB of data was obtained for each sample.

Phylogenetic and Lineage Analysis

The sequencing quality was analyzed with FastQC software v0.11.8. Readouts were filtered and trimmed using the software BBDuk considering a minimum read length of 36 bases and

quality ≥20. Coronavirus assemblies were performed with IRMA software v0.9.3 using as reference NCBI sequence ID NC_045512.2. To identify the G, S, V, and O variants, an alignment was performed using MAFFT v7.458 and Pangolin v1.1.13 package for assigning SARS-CoV-2 genome sequences to global lineage (7).

RESULTS

Geographical Distribution of Variants and Lineages

The full genome analysis of 141 SARS-CoV-2 Chilean cases shows a predominance of the variant G in great part of the

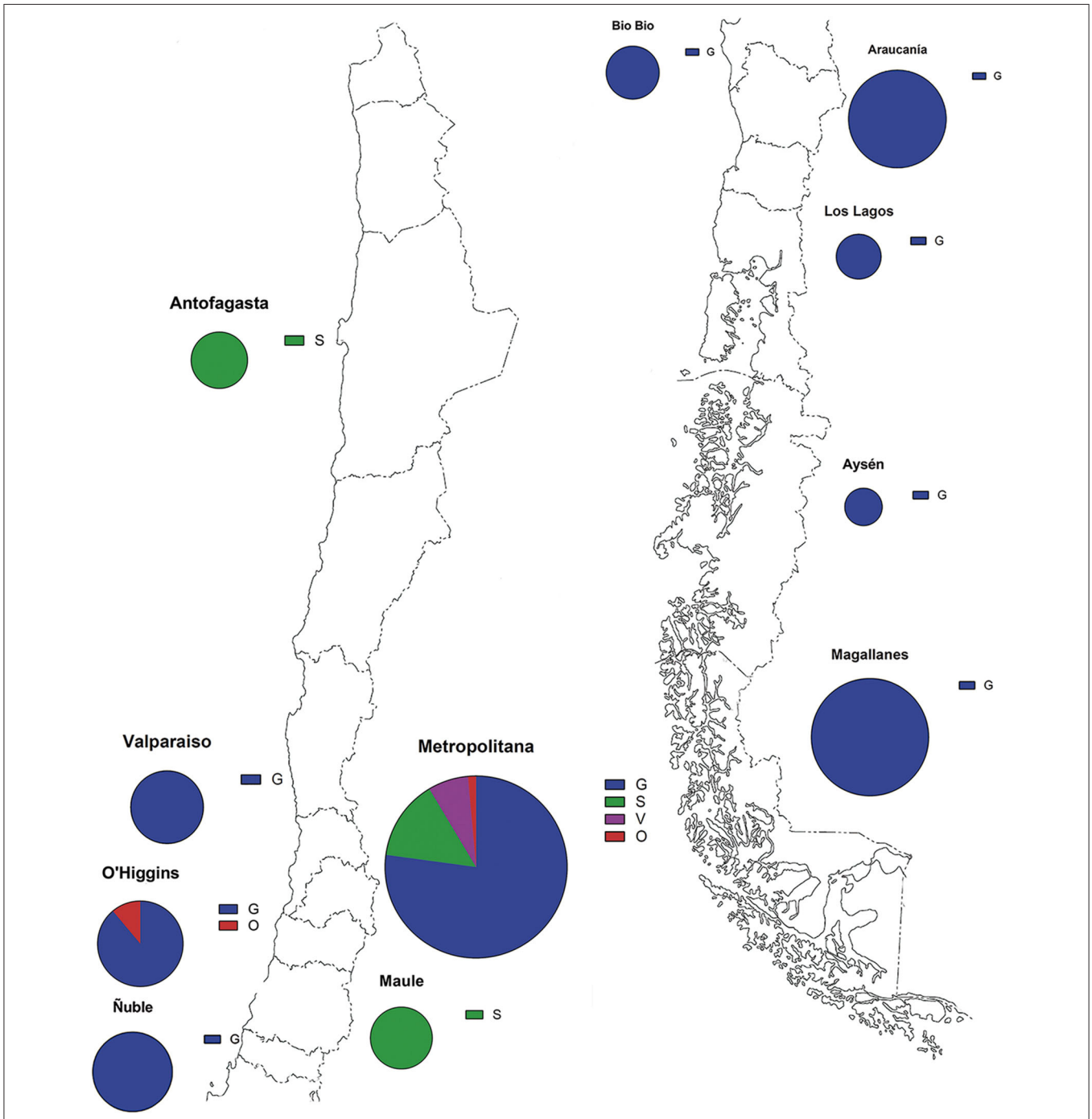


FIGURE 1 | Geographical distribution of the SARS-CoV-2 isolates. Parts of whole graphs represent the proportion of the variants in the regions of Chile. Blue color represents G variant, green S variant, purple V variant, and red O variant. Sphere size is proportional to the number of samples of each zone.

TABLE 1 | Variants and lineages per geographic region in Chile.

Region	Variant	Isolates	Lineage	Isolates
Antofagasta	S	2	A.5	2
	G	5	B.1	5
	G	65	B.1	18
			B.1.1	19
			B.1.1.1	3
			B.1.1.10	2
			B.1.5	22
			B.1.5.4	1
	S	10	A.1	1
			A.2	6
			A.5	3
	V	5	B	1
			B.2	2
			B.2.1	1
			B.2.5	1
	O	1	B	1
O'Higgins	G	8	B.1	1
			B.1.1	5
			B.1.5	2
	O	1	B	1
Maule	S	2	A.5	2
Ñuble	G	4	B.1	3
			B.1.5	1
Bio Bio	G	3	B.1	1
			B.1.1	1
			B.1.5	1
Araucanía	G	14	B.1	13
			B.1.1	1
Los Lagos	G	3	B.1	3
Aysén	G	1	B.1	1
Magallanes	G	17	B.1	17
Total		141		141

TABLE 2 | Nucleotide substitutions associated with viral lineages of Chilean isolates.

Lineage		Position		Region of genome	Protein product
A.2	c	8,782	t	ORF1ab-nsp4	Contains transmembrane domain 2 (TM2)
	t	9,477	a	ORF1ab-nsp4	Contains transmembrane domain 2 (TM2)
	c	14,805	t	ORF1ab-nsp12	RNA-dependent RNA polymerase (RdRp)
	g	25,979	t	ORF3a	ORF3a protein
	t	28,144	c	N	Nucleocapsid nucleoprotein
	c	28,657	t	N	Nucleocapsid nucleoprotein
	c	28,863	t	N	Nucleocapsid nucleoprotein
A.5	c	8,782	t	ORF1ab-nsp4	Contains transmembrane domain 2 (TM2)
	c	26,088	t	ORF3a	ORF3a protein
	t	28,144	c	N	Nucleocapsid nucleoprotein
B.1.1	c	241	t	5′UTR	–
	c	3,037	t	ORF1ab-nsp3	Viral protease
	c	14,408	t	ORF1ab-nsp12	RNA-dependent RNA polymerase (RdRp)
	a	23,403	g	S	Spike protein
	g	28,881	a	N	Nucleocapsid nucleoprotein
	g	28,882	a	N	Nucleocapsid nucleoprotein
	g	28,883	c	N	Nucleocapsid nucleoprotein
B.1.5	c	241	t	5′UTR	–
	c	3,037	t	ORF1ab-nsp3	Viral protease
	c	14,408	t	ORF1ab-nsp12	RNA-dependent RNA polymerase (RdRp)
	a	20,268	g	ORF1ab-nsp15	EndoRNase
	a	23,403	g	S	Spike protein
B.1	c	241	t	5′UTR	–
	c	3,037	t	ORF1ab-nsp3	Viral protease
	c	8,389	t	* ORF1ab-nsp3	Viral protease
	c	14,408	t	ORF1ab-nsp12	RNA-dependent RNA polymerase (RdRp)
	a	23,403	g	**S	Spike protein
	g	25,563	t	ORF3a	ORF3a protein

*SNPs present in the *47.5 and **72% of the samples on this lineage.*

territory between March 2 and April 5, 2020. The Metropolitan region of Santiago houses near to 8.1 million inhabitants and with 17,979 positive cases represents the most sampled area and the second with incidence rate (165.8) second to Magallanes region (437.9) (7). G variant is widespread over the territory, mostly in central and south regions, such as Valparaiso, Metropolitan, O'Higgins, Ñuble, Bio Bio, Araucanía, Los Lagos, Aysén, and Magallanes. S variants are present in the central region of Maule and also in the northern region of Antofagasta. The less represented variants, V and O, were found in the Metropolitan and O'Higgins regions (**Figure 1**, **Table 1**).

According to a recent classification criteria (7), lineages of SARS-CoV-2 can be identified by a phylogenetic analysis and grouped by specific SNPs present in the genome. Lineage B is associated with variants G, V, and O, meanwhile lineage A is related to the variant S. The predominant variant G houses the sublineages B.1, B.1.1, B.1.1.1, B.1.1.10, B.1.5, and B.1.5.4, variant V houses the lineages B, B.2, B.2.1, and B.2.5, and variant O is also associated with the lineage B. On the other hand, variant S is associated with sublineages A.1, A.2, and A.5 (**Table 1**, **Supplementary Figure 1**). A detailed list of the nucleotide substitutions and genome location is presented in **Table 2**.

Progression of the Genetic Variants and Lineages in Chile

The different variants and lineages identified in Chilean samples were analyzed and classified according to the isolate date. Tracing of the genetic mutations allows to identify the progression of the introduced events, local contagion, and the emergence of new sublineages by the introduction of new mutations. In a previous report about the first SARS-CoV-2 complete genome analysis, we detected a relative prevalence of variant S over G, a picture of

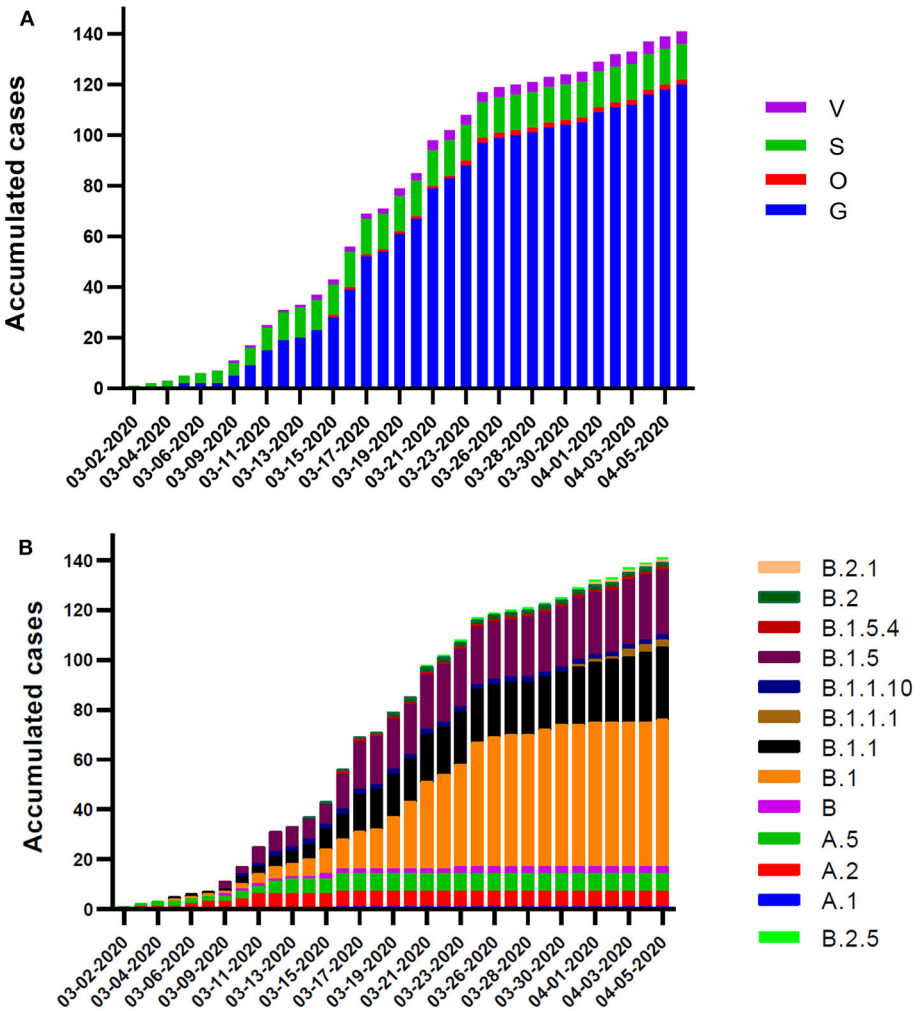


FIGURE 2 | Chronological scheme of the variants and lineages occurrence in Chile. Daily accumulated frequency plot of the sequenced isolates classified according to variant **(A)** and lineage **(B)**. Sampling dates are from March 2 to April 4, 2020.

the beginning of the outbreak in Chile in early March 2020. With the progression of the days and more viruses tested, we observed a rapid amount and predominance of G variant over the wild-type genotype, meanwhile the S variant slightly increased on this period. G variant reaches up to 85.1% of the total samples (120 isolates), followed by S with 10% (14 isolates), V with 3.5% (5 isolates), and O with 1.4% (2 isolates) by the beginning of April 2020 (**Figure 2A**). A similar behavior had the lineage apparition pattern, starting with A.5 (S variant) in early March, and a rapid progression turning predominant sublineages B.1 (41.8%), B.1.1 (20.5%), and B.1.5 (18.4%), all belonging to the G variant (**Figure 2B**).

DISCUSSION

In this work, we present the genetic analysis of the SARS-CoV-2 isolates in Chile, the geographical occurrence of Chilean variants, lineages, and tracking of the outbreak. Current data in all fields of investigation regarding variants and lineages are not sufficient to predict infection rates, host susceptibility, or mortality.

At the beginning of the outbreak, Chinese isolates were mainly variant O, the most related to the reference sequence NC_045512.2, but in mid-January 2020, variant S (T 28144 C) and few cases of V (G 26144 T) and G (A 23403 G) started to appear. By the end of March, the variant O reached 58.6% of the sequenced isolates, followed by S, V, and G with 34, 4.8, and 2.7%, respectively. In the rest of the world, variants distribution changes dramatically compared with China, with special focus in Italy and Spain during March and the USA in April. In European countries, the prevalent variant by the end of March was G (Italy 96.1%, Spain 61%), followed by S (Italy 3.9%, Spain 33.3%), meanwhile in the USA, the most representative variant was also G over S (62.9 and 28.6%) by the end of April, according to the uploaded genomes in the GISAID repository.

We also analyze the variant distribution in South America, and it displays a similar behavior to Spain and the USA,

showing a prevalence of G variant with 74.3%, followed by S, V, and O variants (16.2, 4.8, and 4.8%, respectively). Complete genome sequences were obtained from the GISAID database from Argentina, Brazil, Chile, Colombia, Costa Rica, Ecuador, Mexico, Panama, Peru, and Uruguay between February 25 and April 18.

Beyond the prevalence of certain variants and lineages in South America and Chile compared with the rest of the world, there is not enough evidence to suggest if a particular phenotype is more or less aggressive than others.

The current number of SARS-CoV-2 complete genomes is growing fast every day, but the disease spread is faster. All the collected information regarding genome sequencing represents <1% of the total infected patients (Chile 0.5%, USA 0.38%, Italy 0.04%, Spain 0.18%, China 0.6%); thus, these epidemiological and phylogenetic studies represent the current picture, and the presented data must be considered as that. In the case of Chile, we are collecting samples in more cities in order to generate a genetic landscape from the entire country. In a previous report about the first cases of SARS-CoV-2 in Chile, we described the introduction of the variants S and G from Southeastern Asia and Europe (16), and most of the current cases belong to the G clade, at the beginning only imported cases, but quickly spread into local transmissions.

The pandemic moved to Europe and America after the China outbreak and followed the complete lockdown of countries. G variant quickly spread across every country it took place, reaching more than 50% of the sequenced samples, except in China where it barely rose up to 2.7%. The variant G looks to be more infectious due to its high prevalence over the other variants in the rest of the world but there is still no conclusive evidence to link a unique SNP with the viral phenotype (15). Many other variables are absolutely necessary to consider, such as effective confinement, ethnic groups, access to quality health services, and vaccination programs, in order to confirm/discard those kinds of assumptions (18–21).

Table 2 shows the positions of the SNPs in the genome that determine the variants and lineages and the ORFs where they are located. The most recurrent locations are in the ORF1ab-nsp3, ORF1ab-nsp4, ORF1ab-nsp12 (RdRp), S, and N. Despite the current information and public knowledge about SARS-CoV-2, it is still not possible to determine the precise effect of the nucleotide mutations and the amino acid substitution in viral infectivity, but it is likely that these mutations are involved in differences in viral pathogenesis.

Success in managing the pandemic does not only depend on how the virus is mutating or winning the race to find effective vaccines and antivirals but also depend on how much we have learned from past viral pandemics, how we develop successful social strategies to stop the spread of the virus, and the way we focus our efforts and resources to generate new knowledge by surveillance and high-quality research (22). In this line, we must keep studying viral phylogeny, epidemiology, and molecular and mathematical modeling, and improve diagnostic, and novel and effective therapies (23).

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: www.gisaid.org, Chile.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

AC participated in the conceptualization, study design, interpretation of data analysis, methodology design, and wrote the whole manuscript. BP participated in methodology design and experimental assays. PT participated in genome assemblies, data analysis, and bioinformatics support. JL and LA contributed to genome sequencing. AA, WA, GL, CT, and PB participated in sample processing and real-time RT-PCR assays. RF participated in the critical review of the manuscript. JF contributed to the conceptualization, study design, and critical review of the content and approved the final version of the manuscript. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

The authors are thankful to Vivian Gómez, Jorge Lobos, and all members of the Molecular Genetics and Viral Diseases Sub Departments for their valuable assistance.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2020.562615/full#supplementary-material

REFERENCES

1. Moorthy V, Henao Restrepo AM, Preziosi M, Swaminathan S. Data sharing for novel coronavirus (COVID-19). *Bull World Health.* (2020) 98:150. doi: 10.2471/BLT.20.251561
2. Song P, Karako T. COVID-19: real-time dissemination of scientific information to fight a public health emergency of international concern. *Biosci Trends.* (2020) 14:1–2. doi: 10.5582/bst.2020.01056
3. Heymann DL. Data sharing and outbreaks: best practice exemplified. *Lancet.* (2020) 395:469–70. doi: 10.1016/S0140-6736(20)30184-7
4. World Health Organization W. *WHO Director-General's Opening Remarks at the Media Briefing on COVID-19 - 11 March 2020. WHO Director Generals Speeches.* (2020). Available online at: https://www.who.int/dg/speeches/detail/

who-director-general-s-opening-remarks-at-the-media-briefing-on-covid-19 (accessed March 11, 2020).
5. World Health Organization. *Coronavirus Disease Situation Report-205. World Health Organization*. (2020) 205:1–19. Available online at: https://www.who.int/docs/default-source/coronaviruse/situation-reports/20200812-covid-19-sitrep-205.pdf?sfvrsn=627c9aa8_2 (accessed September 8, 2020).
6. GISAID. *Genomic Epidemiology of hCoV-19*. (2020). Available online at: https://www.epicov.org/epi3/app_entities/entities/corona2020/download_other2.png (accessed May 7, 2020).
7. Rambaut A, Holmes EC, O'Toole A, Hill V, McCrone JT,Ruis C, et al. A dynamic nomenclature for SARS-CoV-2 to assist genomic epidemiology. *Nat Microbiol*. (2020). doi: 10.1038/s41564-020-0770-5. [Epub ahead of print].
8. Duffy S. Why are RNA virus mutation rates so damn high? *PLoS Biol.* (2018) 16:e3000003. doi: 10.1371/journal.pbio.3000003
9. Mandary MB, Masomian M, Poh CL. Impact of RNA virus evolution on quasispecies formation and virulence. *Int J Mol Sci.* (2019) 20:4657. doi: 10.3390/ijms20184657
10. Deng X, Gu W, Federman S, du Plessis L, Pybus OG, Faria NR, et al. Genomic surveillance reveals multiple introductions of SARS-CoV-2 into Northern California. *Science.* (2020) 369:582–7. doi: 10.1126/science.abb9263
11. Phan T. Genetic diversity and evolution of SARS-CoV-2. *Infect Genet Evol.* (2020) 8:104260. doi: 10.1016/j.meegid.2020.104260
12. Pachetti M, Marini B, Benedetti F, Giudici F, Mauro E, Storici P, et al. Emerging SARS - CoV - 2 mutation hot spots include a novel RNA - dependent - RNA polymerase variant. *J Transl Med.* (2020) 18:179. doi: 10.1186/s12967-020-02344-6
13. van Dorp L, Acman M, Richard D, Shaw LP, Ford CE, Ormond L, et al. Emergence of genomic diversity and recurrent mutations in SARS-CoV-2. *Infect Genet Evol.* (2020) 83:104351. doi: 10.1016/j.meegid.2020.104351
14. Korber B, Fischer W, Gnanakaran SG, Yoon H, Theiler J, Abfalterer W, et al. Spike mutation pipeline reveals the emergence of a more transmissible form of SARS-CoV-2. *bioRxiv [preprint].* (2020) 1–33. doi: 10.1101/2020.04.29.069054
15. Korber B, Fischer WM, Gnanakaran S, Yoon H, Theiler J, Abfalterer W, et al. Tracking changes in SARS-CoV-2 Spike: evidence that D614G increases infectivity of the COVID-19 Virus. *Cell.* (2020) 182:812–27.e19. doi: 10.1016/j.cell.2020.06.043
16. Castillo AE, Parra B, Tapia P, Acevedo A, Lagos J, Andrade W, et al. Phylogenetic analysis of the first four SARS-CoV-2 cases in chile. *J Med Virol.* (2020) 19:1562–6. doi: 10.1002/jmv.25797
17. Ministerio de Salud C. Informe epidemiológico N°41. *Minist Salud Chile.* (2020) 41:1–115. Available online at: https://www.minsal.cl/wp-content/uploads/2020/08/Informe-Epidemiologico-41.pdf
18. Miller A, Reandelar MJ, Fasciglione K, Roumenova V, Li Y, Otazu GH. Correlation between universal BCG vaccination policy and reduced mo. *Psychol Appl Work Introd Ind Organ Psychol Tenth Ed Paul.* (2010) 53:1689–99. doi: 10.1101/2020.03.24.20042937
19. Henning-Smith C, Tuttle M, Kozhimannil KB. Unequal distribution of COVID-19 risk among rural residents by race and ethnicity. *J Rural Health.* (2020). doi: 10.1111/jrh.12463. [Epub ahead of print].
20. Pan A, Liu L, Wang C, Guo H, Hao X, Wang Q, et al. Association of public health interventions with the epidemiology of the COVID-19 outbreak in Wuhan, China. *JAMA.* (2020) 323:1873–982. doi: 10.1001/jama.2020.6130
21. Lau H, Khosrawipour V, Kocbach P, Mikolajczyk A, Schubert J, Bania J, et al. The positive impact of lockdown in Wuhan on containing the COVID-19 outbreak in China. *J Travel Med.* (2020) 27:taaa037. doi: 10.1093/jtm/taaa037
22. Ashour HM, Elkhatib WF, Rahman MM, Elshabrawy HA. Insights into the recent 2019 novel coronavirus (SARS-CoV-2) in light of past human coronavirus outbreaks. *Pathogens.* (2020) 9:186. doi: 10.3390/pathogens9030186
23. Ciotti M, Angeletti S, Minieri M, Giovannetti M, Benvenuto D, Pascarella S, et al. COVID-19 outbreak: an overview. *Chemotherapy.* (2020) 64:215–23. doi: 10.1159/000507423

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Public Health

CASE REPORT
published: 22 September 2020
doi: 10.3389/fpubh.2020.00474

Meningitis as an Initial Presentation of COVID-19: A Case Report

Sidra Naz[1†], Muhammad Hanif[2†], Muhammad Adnan Haider[3†], Mukarram Jamat Ali[4†], Muhammad Umer Ahmed[5†] and Sana Saleem[1†]*

[1] Department of Internal Medicine, University of Health Sciences, Lahore, Pakistan, [2] Khyber Medical College Peshawar, Hayatabad Medical Complex, Peshawar, Pakistan, [3] Department of Internal Medicine, Allama Iqbal Medical College, Lahore, Pakistan, [4] Department of Internal Medicine, King Edward Medical University Lahore, Lahore, Pakistan, [5] Ziauddin University and Hospital, Ziauddin Medical College, Karachi, Pakistan

The common presenting symptoms of fever, fatigue, and mild respiratory symptoms like dry cough, are associated with COVID-19, however, patients can also develop neurological manifestations like headache, anosmia, hyposmia, dysgeusia, meningitis, encephalitis, and acute cerebrovascular accidents during the disease. Although very rare, these neurological manifestations are sometimes the sole initial presenting complaint of COVID-19. This case report discusses patients where the initial presenting symptoms seemed to be exclusive to meningitis but the later diagnosis was COVID-19. It is important to increase awareness of these rare presentations in physicians and healthcare workers and facilitate early diagnosis and management to prevent the horizontal spread of the disease.

Keywords: SARS-CoV-2, COVID-19, meningitis, meningo encephalitis, neurological manifestation

OPEN ACCESS

Edited by:
Zisis Kozlakidis, International Agency for Research on Cancer (IARC), France

Reviewed by:
Krishna Nalleballe, University of Arkansas for Medical Sciences, United States
Vasuki Dandu, Baptist Health Foundation, United States

***Correspondence:**
Muhammad Hanif
hanifafridi273@gmail.com

†These authors have contributed equally to this work

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Public Health

Received: *18 June 2020*
Accepted: *27 July 2020*
Published: *22 September 2020*

Citation:
Naz S, Hanif M, Haider MA, Ali MJ, Ahmed MU and Saleem S (2020) Meningitis as an Initial Presentation of COVID-19: A Case Report. Front. Public Health 8:474. doi: 10.3389/fpubh.2020.00474

INTRODUCTION

Following its emergence in Wuhan, China in December 2019, the Coronavirus disease 2019 (COVID-19), which is caused by severe acute respiratory coronavirus-2 (SARS-CoV-2), has become a pandemic (1) and been declared a global health emergency (2). In addition to the common presenting symptoms of fever, fatigue, and mild respiratory symptoms like dry cough, patients with COVID-19 can also develop neurological manifestations like headache, anosmia, hyposmia, dysgeusia, meningitis, encephalitis, and acute cerebrovascular accidents during the course of the disease (3, 4). The first case of meningitis associated with COVID-19 was reported in Japan in February 2020 (5). Since then, two or three more cases of meningoencephalitis have also been reported in the United States (6–8). Although very rare, these neurological manifestations sometimes are the sole initial presenting complaint of COVID-19. In this article, we present a case discussion of instances in which the initial presenting symptoms were exclusive to meningitis and later diagnosed as COVID-19, to make physicians and healthcare workers cognizant of such rare presentations. It is important to diagnose and manage these patients at the earliest possible stage of treatment to prevent the horizontal spread of COVID-19.

CASE REPORT

A 21-years-old male medical student with no known co-morbidities was presented to an emergency department with a 2-days history of frontal headache and fever, and 1-day history of neck stiffness. He denied any cough, shortness of breath, body aches, and diarrhea (**Table 1**). On physical

TABLE 1 | Demographics and clinical characteristics.

Characteristics	Patient
Age	21
Sex	Male
Significant past medical history	None
Symptoms onset	Frontal headache, Fever, and neck stiffness
Respiratory distress	Developed 5 days after symptoms onset
Cause of death	Multi-organ failure

TABLE 2 | Cerebrospinal fluid analysis.

Tests	Results
Appearance	Clear with no xanthochromia
Lactate dehydrogenase	48 U/L
Glucose	83 mg/dL
Protein	164 mg/dL
RBCs	05
Neutrophils	10
Lymphocytes	90
Gram stain/Ziehl-Neelsen Stain	No micro-organisms seen
HSV-PCR	Negative
VZV-PCR	Negative
Culture	No growth after 48 h of incubation

examination, he was alert, oriented, and awake with a Glasgow coma scale score of 15/15. He had a fever of 101 F and neck rigidity with absent Babinski sign and 2+ deep tendon reflexes.

Based on clinical presentations and initial blood work up, bacterial meningitis was suspected and he was started on intravenous antibiotics empirically after cerebral spinal fluid (CSF) was sent for analysis. CSF analysis showed a picture of viral meningitis and in addition to empiric antibiotics, he was also given antiviral agents. CSF gram staining, Ziehl-Neelsen staining, and culture showed no microorganisms, and tests for Herpes simplex type 1, Herpes simplex type 2, and Varicella zoster virus were negative (**Table 2**).

On day 2 of hospitalization (day 4 of initial symptoms), he had swelling of his left eye, and a computed tomography (CT) head was ordered on neurologist recommendation which showed no significant findings. Even though he had no respiratory symptoms of cough and shortness of breath, a chest x-ray was ordered due to the ongoing COVID-19 pandemic and it showed a patch of consolidation. Based on these X-ray findings, testing for COVID-19 was done and a reverse transcriptase polymerase chain reaction for SARS-CoV-2 on nasopharyngeal swab was positive on day 5 of hospitalization. On that same day, he developed tachycardia, tachypnea, and hypotension; his oxygen saturation started to drop progressively and was put on a ventilator. His chest X-ray showed diffuse multi-lobar infiltrates consistent with acute respiratory distress syndrome (**Figure 1**). His laboratory work up showed respiratory acidosis and a picture of disseminated intravascular coagulation (DIC) (**Table 3**). One day later, he passed away due to multi-organ failure.

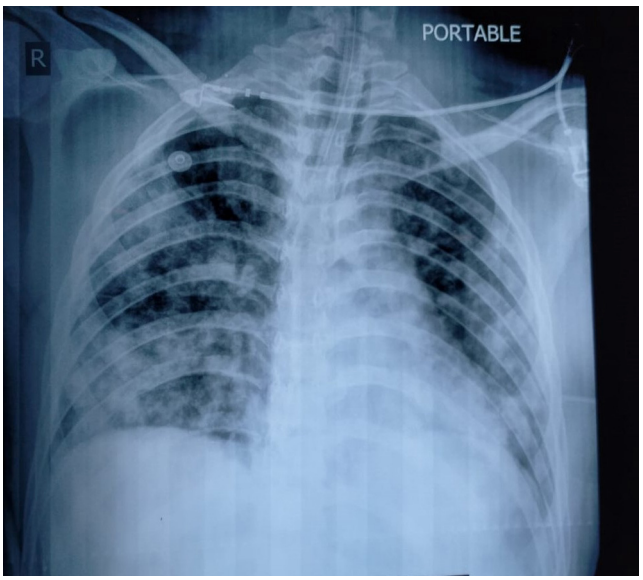


FIGURE 1 | Chest X-ray shows diffuse multi-lobar infiltrates consistent with acute respiratory distress syndrome.

TABLE 3 | Laboratory findings (on day 5 of initial symptoms).

Test	Results
Leukocytes count	2.2 ($x10^9$/l)
Lymphoctes	07%
Neutrophils	89%
Platelet count	65 ($x10^9$/l)
Serum procalcitonin	50 ng/mL
Serum ferritin	1,358 ng/mL
Serum CRP	>32 mg/dL
Serum LDH	527 U/L
Serum CK-MB	54 U/L
Albumin/globulin ratio	0.8
Serum AST	88 U/L
Serum albumin	2.6 g/dL
Serum total protein	5.7 g/dL
International normalized ratio	1.7
Protheombin time	18 s
Plasma FDPs	8,340 ng/FEUm
pH	7.295
HCO_3	22.3 mmol/L
PCO_2	52 mmHg
Base excess/deficit	−2.7 mmol/L

DISCUSSION

This case indicates that in addition to common presenting symptoms of fever, fatigue, and mild respiratory symptoms like dry cough and shortness of breath, patients with COVID-19 can also develop neurological manifestations like headache, anosmia, hyposmia, dysgeusia, meningitis, encephalitis, and

acute cerebrovascular accidents during the course of the disease (3), which highlights the neurotropic potential of SARS-CoV-2 (4). To date, the underlying pathophysiological mechanisms through which SARS-CoV-2 implicates the central nervous system (CNS) are not fully understood, however, the following mechanism have been proposed in other studies (9, 10):

1. Direct spread of SARS-CoV-2 to brain.
2. Spread through neuronal pathways.
3. Haematogenous spread to brain.
4. Immune mediated injury (cytokine storm syndrome).
5. Hypoxic related injury to CNS.

In this case, the patient initially presented with fever and frontal headache along with neck stiffness. There was a delay in the diagnosis because this initial presentation of patients with COVID-19 is rare and only a few cases have been reported so far. To the best of our knowledge, in the USA only two cases have been reported where the initial presenting complaint was a meningitis-like illness (6, 8).

The RBCs in the CSF are an indication of blood brain barrier breach which can occur in SARS-CoV-2 and has been linked to cytokine storm syndrome. Cytokine storm syndrome related damage to the central nervous system has also been implicated in many other viral infections (11). However, only five RBCs in the CSF of our patient could be attributed to traumatic lumbar puncture. Likewise, it has also been suggested that cytokine storm syndrome causes severe symptoms and brain damage in patients with COVID-19 and this is supported by the fact that patients having severe symptoms associated with SARS-CoV-2 infection respond to interleukin-6 (IL-6) receptor blocker [i.e., tocilizumab (12)]. Together with typical picture of viral meningitis on CSF analysis, negative polymerase chain reaction for herpes simplex, and positive reverse transcriptase polymerase chain reaction for SARS-CoV-2 on a nasopharyngeal swab, we labeled this case of meningitis as viral meningitis secondary to SARS-CoV-2. Although there have been some cases reported of SARS-CoV-2 detection in CSF by reverse transcriptase polymerase chain reaction (RT-PCR) (5, 13), yet US Food and Drug Administration has not approved any test to detect SARS-CoV-2 in CSF. Additionally, as the CT head was normal in this case, we should have ordered a magnetic resonance imaging of the brain for detailed imaging.

During this ongoing pandemic, there is a need to make physicians and other healthcare workers cognizant of rare presentations such as this, so that we can diagnose and manage these patients at the earliest possible opportunity, which prevents the horizontal spread of the virus and ensures patient safety. We recommend testing of CSF for SARS-CoV-2 *via* RT-PCR in suspected cases.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

REFERENCES

1. Lu H, Stratton CW, Tang YW. Outbreak of pneumonia of unknown etiology in Wuhan, China: the mystery and the miracle. *J Med Virol.* (2020) 92:401–2. doi: 10.1002/jmv.25678
2. Wang C, Horby PW, Hayden FG, Gao GF. A novel coronavirus outbreak of global health concern. *Lancet*. (2020) 395:470–3. doi: 10.1016/S0140-6736(20)30185-9
3. Mao L, Jin H, Wang M, Hu Y, Chen S, He Q, et al. Neurologic manifestations of hospitalized patients with coronavirus disease 2019 in Wuhan, China. *JAMA Neurol*. (2020) 77:1–9. doi: 10.1001/jamaneurol.2020.1127
4. Ahmed MU, Hanif M, Ali MJ, Haider MA, Kherani D, Memon GM, et al. Neurological manifestations of COVID-19 (SARS-CoV-2): a review. *Front Neurol.* (2020) 11:518. doi: 10.3389/fneur.2020.00518
5. Moriguchi T, Harii N, Goto J, Harada D, Sugawara H, Takamino J, et al. A first case of meningitis/encephalitis associated with SARS-Coronavirus-2. *Int J Infect Dis.* (2020) 94:55–8. doi: 10.1016/j.ijid.2020.03.062
6. Duong L, Xu P, Liu A. Meningoencephalitis without respiratory failure in a young female patient with COVID-19 infection in Downtown Los Angeles, early April 2020. *Brain Behav Immun.* (2020) 87:83. doi: 10.1016/j.bbi.2020.04.024
7. Poyiadji N, Shahin G, Noujaim D, Stone M, Patel S, Griffith B. COVID-19-associated acute hemorrhagic necrotizing encephalopathy: CT and MRI features. *Radiology*. (2020) 296:201187. doi: 10.1148/radiol.2020201187
8. Packwood R, Galletta G, Tennyson J. An unusual case report of COVID-19 presenting with meningitis symptoms and shingles. *Clin Practice Cases Emergency Med*. (2020) 4:316–20. doi: 10.5811/cpcem.2020.4.47557
9. Zhao Y, Zhao Z, Wang Y, et al. Single-cell RNA expression profiling of ACE2, the putative receptor of Wuhan 2019-nCov. *bioRxiv*. (2020). doi: 10.1101/2020.01.26.919985
10. Baig AM, Khaleeq A, Ali U, Syeda H. Evidence of the COVID-19 virus targeting the CNS: tissue distribution, host–virus interaction, and proposed neurotropic mechanisms. *ACS Chem. Neurosci.* 11:995–8. doi: 10.1021/acschemneuro.0c00122
11. Popescu CP, Florescu SA, Lupulescu E, Zaharia M, Tardei G, Lazar M, et al. Neurologic complications of influenza B virus infection in adults, Romania. *Emerg Infect Dis.* (2017) 23:574–81. doi: 10.3201/eid2304.161317
12. Zhang C, Wu Z, Li JW, Zhao H, Wang GQ. Cytokine release syndrome in severe COVID-19: interleukin-6 receptor antagonist tocilizumab may be the key to reduce mortality. *Int J Antimicrob Agents.* (2020) 55:105954. doi: 10.1016/j.ijantimicag.2020.105954

13. Huang YH, Jiang D, Huang JT. SARS-CoV-2 detected in cerebrospinal fluid by PCR in a case of COVID-19 encephalitis. *Brain Behav Immun.* (2020) 87:149. doi: 10.1016/j.bbi.2020.05.012

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 23 September 2020
doi: 10.3389/fimmu.2020.560381

Increased Serum Levels of sCD14 and sCD163 Indicate a Preponderant Role for Monocytes in COVID-19 Immunopathology

Jose Gómez-Rial[1,2†], Maria José Currás-Tuala[1†], Irene Rivero-Calle[1,3], Alberto Gómez-Carballa[1,4], Miriam Cebey-López[1], Carmen Rodríguez-Tenreiro[1], Ana Dacosta-Urbieta[1,3], Carmen Rivero-Velasco[5], Nuria Rodríguez-Núñez[6], Rocio Trastoy-Pena[7], Javier Rodríguez-García[8], Antonio Salas[1,4] and Federico Martinón-Torres[1,3*]*

[1] Grupo de Investigación en Genética, Vacunas, Infecciones y Pediatría, Instituto de Investigación Sanitaria de Santiago, Hospital Clinico Universitario and Universidade de Santiago de Compostela, Servizo Galego de Saúde, Galicia, Spain, [2] Laboratorio de Inmunologìa, Servicio de Análisis Clìnicos, Hospital Clinico Universitario Santiago de Compostela, Servizo Galego de Saúde, Galicia, Spain, [3] Translational Pediatrics and Infectious Diseases Section, Department of Pediatrics, Hospital Clìnico Universitario de Santiago de Compostela, Galicia, Spain, [4] Unidade de Xenética, Instituto de Ciencias Forenses, Facultade de Medicina, Universidade de Santiago de Compostela, and GenPoB Research Group, Instituto de Investigación Sanitaria (IDIS), Hospital Clìnico Universitario de Santiago, Servizo Galego de Saúde, Galicia, Spain, [5] Intensive Medicine Department, Hospital Clìnico Universitario de Santiago de Compostela, Galicia, Spain, [6] Pneumology Department, Hospital Clìnico Universitario de Santiago de Compostela, Galicia, Spain, [7] Microbiology Department, Hospital Clìnico Universitario de Santiago de Compostela, Galicia, Spain, [8] Clinical Biochemistry Laboratory, Hospital Clìnico Universitario de Santiago de Compostela, Galicia, Spain

OPEN ACCESS

Edited by:
Annalisa Del Prete, University of Brescia, Italy

Reviewed by:
Angela Ceribelli, Humanitas Research Hospital, Italy
Kenneth Williams, Boston College, United States

****Correspondence:***
Jose Gómez-Rial
jose.gomez.rial@sergas.es
Federico Martinón-Torres
federico.martinon.torres@sergas.es

[†] These authors have contributed equally to this work

Specialty section:
This article was submitted to Cytokines and Soluble Mediators in Immunity, a section of the journal Frontiers in Immunology

Received: *08 May 2020*
Accepted: *02 September 2020*
Published: *23 September 2020*

Citation:
Gómez-Rial J, Currás-Tuala MJ, Rivero-Calle I, Gómez-Carballa A, Cebey-López M, Rodríguez-Tenreiro C, Dacosta-Urbieta A, Rivero-Velasco C, Rodríguez-Núñez N, Trastoy-Pena R, Rodríguez-García J, Salas A and Martinón-Torres F (2020) Increased Serum Levels of sCD14 and sCD163 Indicate a Preponderant Role for Monocytes in COVID-19 Immunopathology. Front. Immunol. 11:560381. doi: 10.3389/fimmu.2020.560381

Background: Emerging evidence indicates a potential role for monocytes in COVID-19 immunopathology. We investigated two soluble markers of monocyte activation, sCD14 and sCD163, in COVID-19 patients, with the aim of characterizing their potential role in monocyte-macrophage disease immunopathology. To the best of our knowledge, this is the first study of its kind.

Methods: Fifty-nine SARS-Cov-2 positive hospitalized patients, classified according to ICU or non-ICU admission requirement, were prospectively recruited and analyzed by ELISA for levels of sCD14 and sCD163, along with other laboratory parameters, and compared to a healthy control group.

Results: sCD14 and sCD163 levels were significantly higher among COVID-19 patients, independently of ICU admission requirement, compared to the control group. We found a significant correlation between sCD14 levels and other inflammatory markers, particularly Interleukin-6, in the non-ICU patients group. sCD163 showed a moderate positive correlation with the time lapsed from admission to sampling, independently of severity group. Treatment with corticoids showed an interference with sCD14 levels, whereas hydroxychloroquine and tocilizumab did not.

Conclusions: Monocyte-macrophage activation markers are increased and correlate with other inflammatory markers in SARS-Cov-2 infection, in association to hospital admission. These data suggest a preponderant role for monocyte-macrophage activation in the development of immunopathology of COVID-19 patients.

Keywords: COVID-19, monocyte, sCD14, sCD163, immunopathology

INTRODUCTION

Emerging evidence from SARS-Cov-2 infected patients suggests a key role for monocyte-macrophage in the immunopathology of COVID-19 infection, with a predominant monocyte-derived macrophage infiltration observed in severely damaged lungs (1), and morphological and inflammation-related changes in peripheral blood monocytes that correlate with the patients' outcome (2). An overexuberant inflammatory immune response with production of a cytokine storm and T-cell immunosuppression are the main hallmarks of severity in these patients (3). This clinical course resembles viral-associated hemophagocytic syndrome (VAHS), a rare severe complication of various viral infections mediated by proinflammatory cytokines, resulting in multiorgan failure and death (4). A chronic expansion of inflammatory monocytes and over-activation of macrophages have been extensively described in this syndrome (5–7). Viral-associated hemophagocytic syndrome has been identified as a major contributor to death of patients in past pandemics caused by coronaviruses (8), including previous SARS and MERS outbreaks (9), and currently suggested for SARS-Cov-2 outbreak (10).

CD14 and CD163 are both myeloid differentiation markers found primarily on monocytes and macrophages, and detection of soluble release of both in plasma is considered a good biomarker of monocyte-macrophage activation (11, 12). Elevated plasma levels of soluble CD14 (sCD14) are associated to poor prognosis in VIH-infected patients, are a strong predictor of morbidity and mortality (13, 14), and associated with diminished CD4+-T cell restoration (15). In addition, soluble CD163 (sCD163) plasma levels are a good proxy for monocyte expansion and disease progression during HIV infection (16). In measles infection, a leading cause of death associated with increased susceptibility to secondary infections and immunosuppression, sCD14 and sCD163 levels have been found to be significantly higher, indicating an important and persistent monocyte-macrophage activation (17).

We hypothesized that monocytes/macrophages may be an important component of immunopathology associated to SARS-Cov-2 infection. In this paper, we analyze serum levels of soluble monocyte activation markers in COVID-19 patients and their correlation with severity and other inflammatory markers.

MATERIALS AND METHODS

Subjects

We recruited 59 patients with confirmed PCR-positive diagnosis of SARS-Cov-2 infection, classified according to ICU admission requirement ($n = 22$ patients), or non-ICU requirement ($n = 37$), and age-matched healthy individuals ($n = 20$) as a control group. Demographic data, main medication treatment and routine lab clinical parameters including inflammatory biomarkers were collected for all infected patients. Leftover sera samples from routine analytical controls were employed for the analysis, after obtaining the corresponding informed consent. Time elapsed from hospital admission to sample extraction was also recorded.

Measurement of sCD14 and sCD163 Serum Levels

To determine levels of soluble monocyte activation markers in serum specimens, appropriate sandwich ELISA (Quantikine, R&D systems, United Kingdom) were used following manufacturer indications. Briefly, diluted sera samples were incubated for 3 h at room temperature in the corresponding microplate strips coated with capture antibody. After incubation, strips were washed and incubated with the corresponding Human Antibody conjugate for 1 h. After washing, reactions were revealed and optical density at 450 nm was determined in a microplate reader. Concentration levels were interpolated from the standard curve using a four-parameter logistic (4-PL) curve-fit in Prism8 GraphPad software. Final values were corrected applying the corresponding dilution factor employed.

Statistical Analysis

Data are expressed as median and interquartile range. All statistical analyses were performed using the statistical package R. Mann–Whitney tests were used for comparison between ICU and non-ICU groups *versus* healthy controls. Pearson's correlation coefficients were used to quantify the association between sCD14 and sCD163 concentration and other lab parameters in non-ICU patients. Data outliers, falling outside the 1.5 interquartile range, were excluded from the statistical analysis. The nominal significance level considered was 0.05. Bonferroni adjustment was used to account for multiple testing.

RESULTS

Demographic and Clinical Laboratory Parameters

Patients in the ICU group showed significant differences when compared to non-ICU group in several clinical laboratory parameters: lymphocytes, ferritin, D-dimer, Lactate dehydrogenase (LDH), procalcitonin (PCT), and Interleukin-6 (IL-6). The absolute value for circulating monocytes did not show significant differences between groups. However, these values may have been distorted by the use of tocilizumab, an IL-6 blocking drug extensively employed in the ICU group which interferes with monocyte function. Age and time elapsed from admission to sample extraction did not show differences between groups. Values are summarized in **Table 1**.

Serum Levels for sCD14 and sCD163

Median levels for sCD14 in sera from ICU patients were 2444.0 (95%CI: 1914.0–3251.0) ng/ml, compared to 2613.0 (95%CI: 2266.0–2991.0) ng/ml in non-ICU patients. The healthy control group median value was 1788.0 (95%CI: 1615.0–1917.0) ng/ml. We observed significant statistical differences when comparing infected patients against controls (P-value < 0.0001), however no significant differences were observed between ICU and non-ICU

TABLE 1 | Demographic and clinical laboratory parameters of patients recruited.

Parameter	ICU	non-ICU	*P*-value
Clinical laboratory parameters			
Lymphocytes	0.54 (0.47–1.058)	1.16 (0.79–1.62)	**0.0004**
Monocytes	0.35 (0.16–0.65)	0,42 (0.35–0.58)	ns
Platelets	264 (204.3–354.5)	272 (213–413)	ns
D-Dimer	3676 (1198–8121)	755 (413–1033)	**0.0002**
Lactate dehydrogenase (LDH)	677 (429–818.5)	469 (391–595)	**0.0188**
C-reactive protein (CRP)	7.37 (2.56–20.51)	4,65 (2.16–11.41)	ns
Procalcitonin (PCT)	0.22 (0.09–0.4)	0.09 (0.05–0.21)	**0.0305**
Ferritin	1257 (837.3–3020)	467 (254.5–785)	**<0.0001**
Interleukin-6 (IL-6)	83.10 (14.45–381.8)	12.70 (6.95–46)	**0.0014**
Glycosylated hemoglobin (Hb1Ac)	5.95 (5.65–6.47)	6.1 (5.7–6.9)	ns
Troponin-I	0.021 (0.017–0.246)	0.017 (0.017–0.019)	ns
Time elapsed from admission to sample (days)			
	5 (3.75–10)	4 (2–6)	ns
Age (years)			
	52 (48.75–61.25)	52 (44–65)	ns
Corticoids			
	19/22 (87%)	2/37 (5.4%)	**<0.0001**

Bold values are significant values.

TABLE 2 | Concentration (ng/ml) of serum levels of sCD14 and sCD163 in patients from ICU and non-ICU groups, and healthy controls.

Concentration	ICU	non-ICU	Healthy controls
sCD14	2444.0 (1914.0–3251.0)	2613.0 (2266.0–2991.0)	1788.0 (1615.0–1917.0)
sCD163	911.5 (624.7–1167)	910.4 (733.1–1088)	495.6 (332.5–600.7)

Data are represented as median and interquartile range.

groups. Median levels for sCD163 in sera from ICU patients were 911.5 (95%CI: 624.7–1167.0) ng/ml, and 910.4 (95%CI: 733.1–1088.0) ng/ml in non-ICU patients. The healthy control group value was 495.6 (95%CI: 332.5–600.7) ng/ml. As with sCD14, we observed significant differences for values from infected patients compared to control group (P-value < 00001), but no differences between ICU and non-ICU infected patients. Values are summarized in **Table 2** and **Figure 1**.

Correlation Between sCD14 and sCD163 Levels and Time Elapsed From Hospital Admission

We assessed the correlation between sCD14 and sCD163 levels and time elapsed from hospital admission to sample extraction (**Figure 2**). We found a significant positive correlation between sCD163 levels and time elapsed (r^2 = 0.3246, P-value = 0.0156) We did not observe a significant correlation between sCD14 levels and time elapsed from hospital admission to sample extraction.

Correlation Between sCD14 and sCD163 Levels and Clinical Laboratory Parameters

We found significant correlations between sCD14 and sCD163 levels and several clinical laboratory parameters in infected patients (in these analysis, adjusted significance under Bonferrori correction is 0.01), but only in the non-ICU group, possibly reflecting an interference of the use of tocilizumab or corticoids in the ICU group. Levels of sCD14 showed a negative correlation with the absolute value of lymphocytes (r^2 = −0.5501, P-value = 0.0005) and a positive correlation with levels of LDH (r^2 = 0.5906, P-value = 0.0001), CRP (r^2 = 0.6275, P-value < 0.0001); PCT (r^2 = 0.4608, P-value = 0.0091), and Ferritin (r^2 = 0.4414, P-value = 0.0090) (**Figure 3**). No other significative associations were found with other lab parameters. Levels of sCD163 did not show significant correlation with clinical laboratory parameters (**Figure 3**). Particularly, IL-6 also showed significant positive correlation with sCD14 (r^2 = 0.6034, P-value = 0.0003) (**Figure 4**).

Effect of Treatment on sCD14 and sCD163 Levels

We analyzed possible interference of different treatments on sCD14 and sCD163 serum levels for all patients. We found an interference of corticoid treatment on sCD14, levels with median values of 2034 (95%CI: 1319–3159) ng/ml for treated group, and values of 2613 (95%CI: 2466–2913) ng/ml for non-treated group. Values were significantly lower in corticoid-treated group (P-value = 0.0069) (**Figure 5**). No impact was found for corticoids on sCD163 levels. Likewise, hydroxychloroquine and/or tocilizumab were not found to have an impact on sCD14 and sCD163 serum levels.

Correlation Between sCD14 and sCD163 Levels and Hospital Stay

Levels of sCD14 and sCD163 did not show association with length of hospital stay in both groups. Also, these biomarkers did not show association with the number of days of onset of symptoms.

Age-Dependence of sCD14 and sCD163 Levels

We analyzed for possible age-dependence of sCD14 and sCD163 levels. Values did not show association between these biomarker levels and the age of patients.

DISCUSSION

Our results show, for the first time, increased levels of sCD14 and sCD163 in sera from SARS-Cov-2 infected patients admitted to hospital. We did not observe statistical differences when comparing ICU versus non-ICU patients. This is probably due

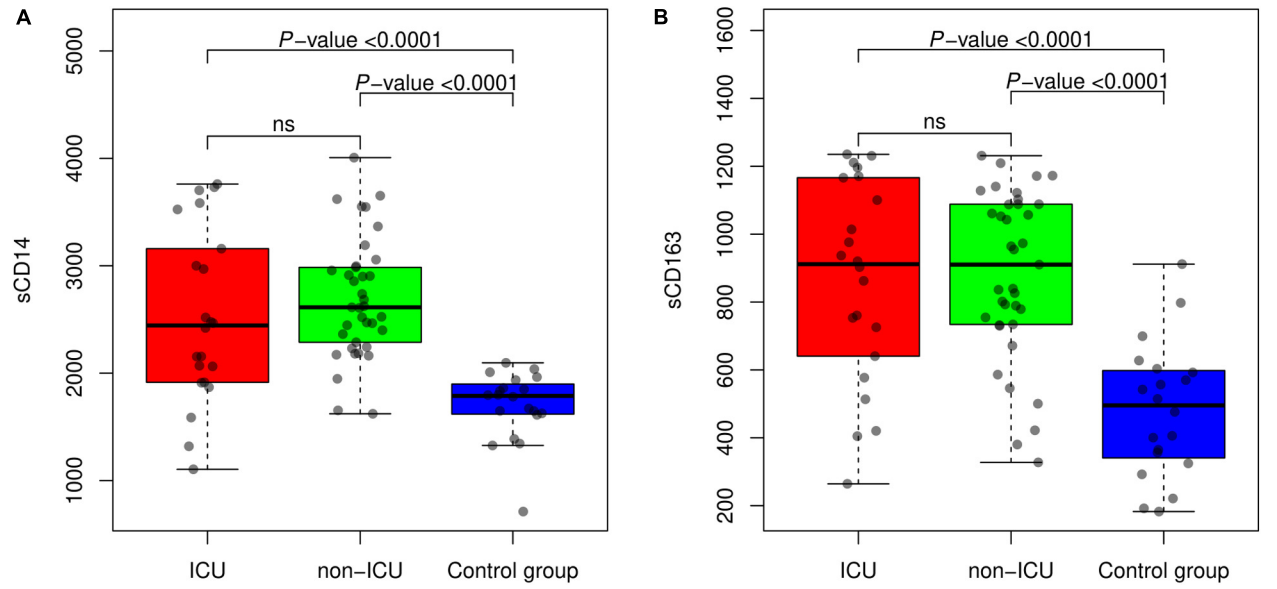


FIGURE 1 | Values of sCD14 **(A)** and sCD163 **(B)** in sera samples from patients in ICU, non-ICU, and healthy controls. Results are presented as median and interquartile range levels in ng/ml. Non-parametric Mann–Whitney tests were used for comparison between groups, and *P*-values for the different comparisons are displayed.

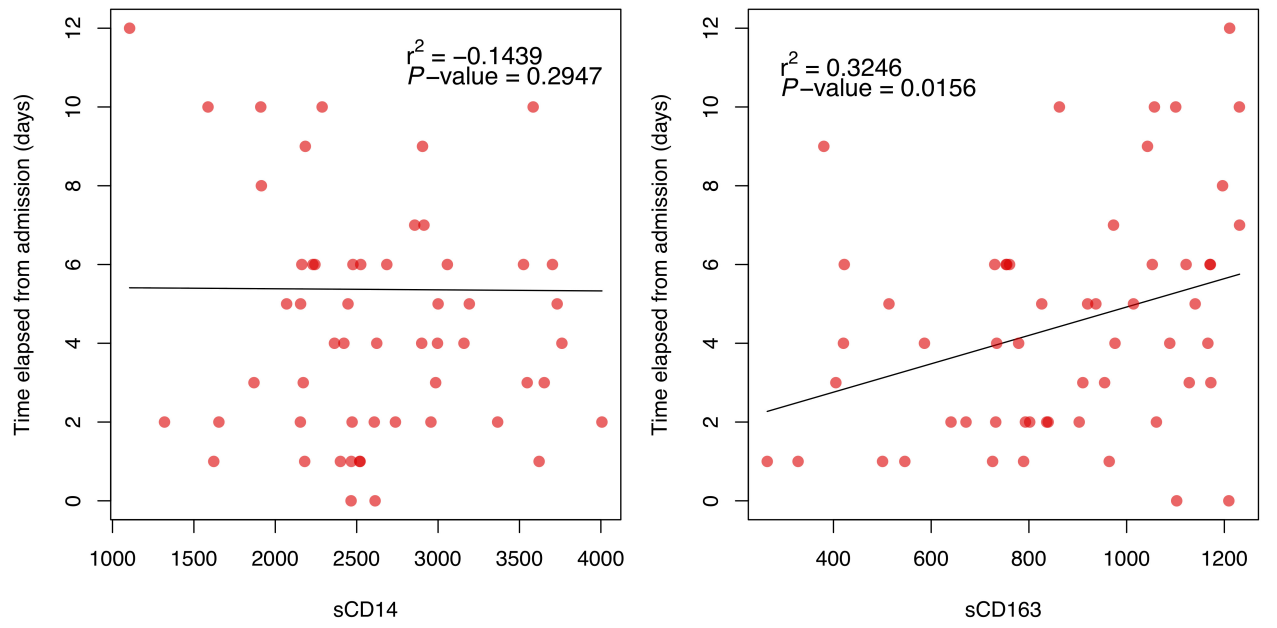


FIGURE 2 | Correlation between serum levels of sCD14 and sCD163 and time elapsed from admission to sample extraction (in days) for all infected patients. Pearson's correlation coefficient (r^2) and *P*-value are shown.

to the interference on monocyte function and sCD14 levels produced by the use of corticoid treatment in ICU patients, as shown here and previously by others (18, 19). However, levels of sCD14 showed a strong correlation with clinical laboratory parameters, including acute phase reactants (ferritin, LDH, C-reactive protein, procalcitonin) and a strong correlation with IL-6 levels in the non-ICU patient group, where no corticoids treatments were used. Hydroxychloroquine and tocilizumab treatment did not show interferences on sCD14 and sCD163 levels. Furthermore, sCD163 levels showed a correlation with the time elapsed from hospital admission to sample extraction, suggesting a potential indicator of disease progression.

Monocytes and macrophages constitute a key component of immune responses against viruses, acting as bridge between

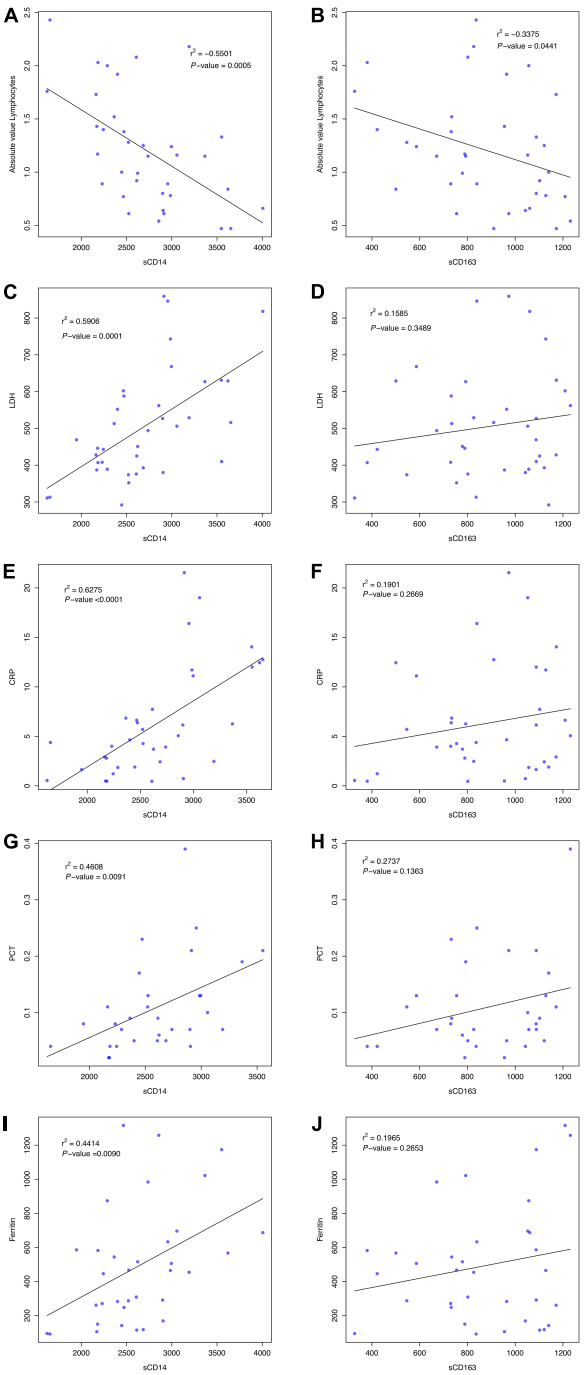


FIGURE 3 | Association between serum levels of sCD14 and sCD163, and several laboratory parameters including Absolute Valor Lymphocytes **(A,B)**, LDH **(C,D)**, CRP **(E,F)**, PCT **(G,H)**, and Ferritin **(I,J)** in the non-ICU patient group. Pearson's correlation coefficient (r^2) and *P*-value are shown. LDH, lactate dehydrogenase; CRP, C-reactive protein; PCT, procalcitonin.

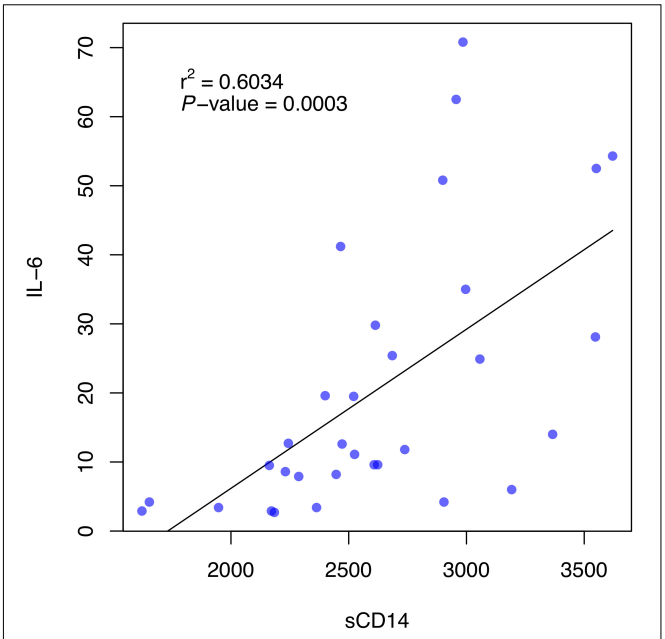


FIGURE 4 | Association between serum levels of sCD14 and IL-6 levels in the non-ICU patient group. Pearson's correlation coefficient (r^2) and *P*-value are presented.

innate and adaptive immunity (20). Activation of macrophages has been demonstrated to be pivotal in the pathogenesis of the immunosuppression associated to several viral infections (such as VIH, measles), where expansion of specific subsets of monocytes and macrophages in peripheral blood are observed, and considered to be drivers of immunopathogenesis (21). Our results support the hypothesis of a preponderant role for monocytes in SARS-Cov-2 immunopathology, associated to an overexuberant immune response. Increased levels of monocyte-macrophage activation markers, and their correlation with other inflammatory biomarkers (particularly IL-6), indicate a close relationship between monocyte activation and immunopathology in these patients. Inflammatory markers are closely related to severity in COVID-19 pathology (22) and selective blockade of IL-6 has been demonstrated to be a good therapeutic strategy in COVID-19 pathology (23). Our results thus suggest that monocyte-macrophage activation can act as driver cells of the cytokine storm and immunopathology associated to severe clinical course of COVID-19 patients. Further, monitorization of monocyte activity trough these soluble activation markers and/or follow-up of circulating inflammatory monocytes in peripheral blood, could be useful to assess disease progression in the same way as in other viral infections (16).

In addition, our results identify monocyte-macrophage as a good target for the design of therapeutic intervention using drugs that inhibit monocyte-macrophage activation and differentiation. In this sense, anti-GM CSF inhibitor drugs, currently under clinical trials for rheumatic and other auto-inflammatory diseases, might provide satisfactory results in COVID-19 patients. Other drugs targeting monocyte and/or macrophage could also be useful in COVID-19, as in other inflammatory diseases (24). The strategy of inhibiting monocyte differentiation has proved useful in avoiding cytokine storm syndrome after CAR-T cell immunotherapy (25), suggesting a possible therapeutic application to COVID-19 immunopathology (26, 27).

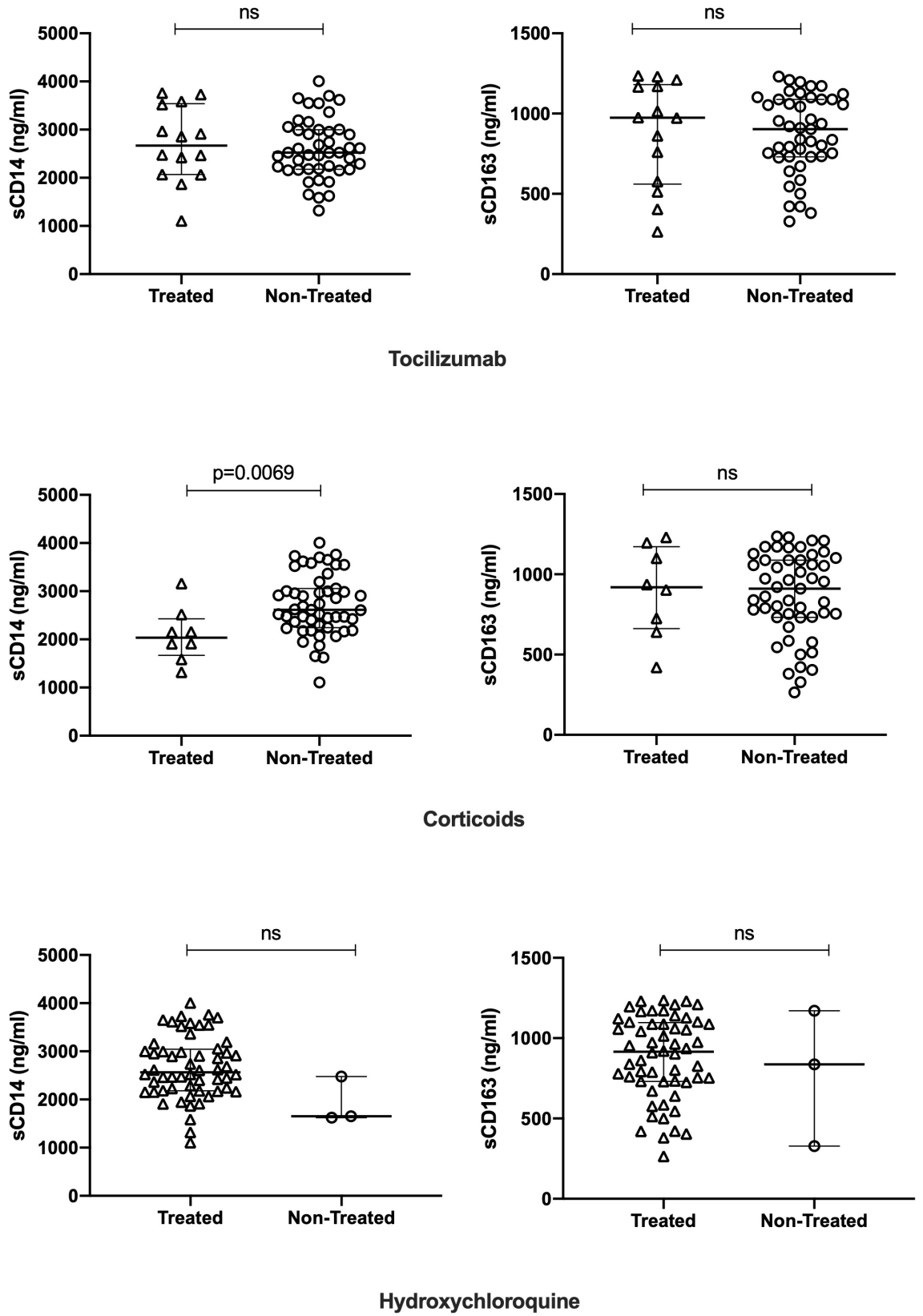


FIGURE 5 | Effects of corticoid, hydroxychloroquine, and tocilizumab treatment on sCD14 and sCD163 levels. Results are presented as median and interquartile range levels in ng/ml. Non-parametric Mann–Whitney tests were used for comparison between groups, and *P*-values for the different comparisons are displayed.

The present study has several limitations, including a relatively low sample size and the interference of corticoids in ICU patients' results. However, these preliminary results are strongly suggestive of an important implication of monocyte-macrophage in COVID-19 immunopathology, as highlighted by the correlations found between these biomarker levels and inflammatory parameters. Further studies using broader series are needed to confirm our findings.

In summary, our data underscore the preponderant role of monocyte and macrophage immune response in COVID-19 immunopathology and provide pointers for future interventions in drug strategies and monitoring plans for these patients.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comité de Ética de la Investigación con Medicamentos de Galicia (fast-track approval 18-march-2020). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

JG-R, FM-T, and AS designed and conceptualized the study and made the first draft. MC-T, IR-C, AG-C, MC-L, CR-T, AD-U, CR-V, NR-N, RT-P, and JR-G collected the samples and did the analysis, reviewed the draft and approved the final version. All authors contributed to the article and approved the submitted version.

FUNDING

This study received support from the Instituto de Salud Carlos III: project GePEM (Instituto de Salud Carlos III(ISCIII)/PI16/01478/Cofinanciado FEDER), DIAVIR (Instituto de Salud Carlos III(ISCIII)/DTS19/00049/Cofinanciado FEDER; Proyecto de Desarrollo Tecnológico en Salud) and Resvi-Omics (Instituto de Salud Carlos III(ISCIII)/PI19/01039/Cofinanciado FEDER) and project BI-BACVIR (PRIS-3; Agencia de Conocimiento en Salud (ACIS)—Servicio Gallego de Salud (SERGAS)—Xunta de Galicia; Spain) given to AS; and projects ReSVinext (Instituto de Salud Carlos III(ISCIII)/PI16/01569/Cofinanciado FEDER), and Enterogen (Instituto de Salud Carlos III(ISCIII)/PI19/01090/Cofinanciado FEDER) given to FM-T.

ACKNOWLEDGMENTS

This work was developed with support from the GEN-COVID Study Group (www.gencovid.eu) from the Hospital Clínico Universitario de Santiago de Compostela, Instituto de Investigación Sanitaria de Santiago and Universidad de Santiago de Compostela, whose members are: Aguilera Guirao, Antonio; Álvarez Escudero, Julián; Antela López, Antonio; Barbeito Castiñeiras, Gema; Bello Paderne, Xabier; Ben García, Miriam; Carral García, María Victoria; Cebey López, Miriam; Coira Nieto, Amparo; Conde Pájaro, Mónica; Costa Alcalde, José Javier; Currás Tuala, María José; Dacosta Urbieta, Ana Isabel; Díaz Esteban, Blanca; Domínguez Santalla, María Jesús; Fernández Villaverde, Juan; Galbán Rodríguez, Cristóbal; García Allut, José Luis; García Vicente, Luisa; Giráldez Vázquez, Elena; Gómez Carballa, Alberto[1]; Gómez Rial, José; González Barcala, Francisco Javier; Guerra Liñares, Beatriz; Leboráns Iglesias, Pilar; Lence Massa, Beatriz; Lendoiro Fuentes, Marta; López Franco, Montserrat; López Lago, Ana; Navarro De la Cruz, Daniel; Núñez Masid, Eloína; Ortolá Devesa, Juan Bautista; Pardo Seco, Jacobo; Pazo Núñez, María; Pérez del Molino Bernal, Marisa; Pérez Freixo, Hugo; Piñeiro Rodríguez, Lidia; Pischedda, Sara; Portela Romero, Manuel; Pose Reino, Antonio; Prada Hervella, Gloria María; Queiro Verdes, Teresa; Redondo Collazo, Lorenzo; Regueiro Casuso, Patricia; Rey García, Susana; Rey Vázquez, Sara; Riveiro Blanco, Vanessa; Rivero Calle, Irene; Rivero Velasco, Carmen; Rodríguez Núñez, Nuria; Rodríguez-Tenreiro Sánchez, Carmen; Saborido Paz, Eva; Sadiki Orayyou, José Miguel; Saito Villanueva, Carla; Serén Fernández, Sonia; Souto Sanmartín, Pablo; Taboada Muñiz, Manuel; Trastoy Pena, Rocío; Treviño Castellano, Mercedes; Valdés Cuadrado, Luis; Varela García, Pablo; Vilas Iglesias, María Soledad; Viz Lasheras, Sandra. We would like to acknowledge the effort of all first-line healthcare workers supporting the patients included in this study.

REFERENCES

1. Liao M, Liu Y, Yuan J, Wen Y, Xu G, Zhao J, et al. The landscape of lung bronchoalveolar immune cells in COVID-19 revealed by single-cell RNA sequencing. *medRxiv*. (2020). [Preprint]. doi: 10.1101/2020.02.23.20026690
2. Zhang D, Guo R, Lei L, Liu H, Wang Y, Wang Y, et al. COVID-19 infection induces readily detectable morphological and inflammation-related phenotypic changes in peripheral blood monocytes, the severity of wich correlate with patient outcome. *medRxiv*. (2020). [Preprint]. doi: 10.1101/2020.03.24.20042655
3. Mehta P, McAuley DF, Brown M, Sanchez E, Tattersall RS, Manson JJ, et al. Speciality Collaboration, COVID-19: consider cytokine storm syndromes and immunosuppression. *Lancet*. (2020) 395:1033–4. doi: 10.1016/S0140-6736(20)30628-0
4. McKenna RW, Risdall RJ, Brunning RD. Virus associated hemophagocytic syndrome. *Hum Pathol*. (1981) 12:395–8. doi: 10.1016/S0046-8177(81)80019-6
5. Emminger W, Zlabinger GJ, Fritsch G, Urbanek R. CD14(dim)/CD16(bright) monocytes in hemophagocytic lymphohistiocytosis. *Eur J Immunol*. (2001) 31:1716–9. doi: 10.1002/1521-4141(200106)31:6<1716::AID-IMMU1716>3.0.CO;2-X
6. Brisse E, Wouters CH, Andrei G, Matthys P. How viruses contribute to the pathogenesis of hemophagocytic lymphohistiocytosis. *Front Immunol*. (2017) 8:1102. doi: 10.3389/fimmu.2017.01102

7. La Rosee P, Horne A, Hines M, von Bahr Greenwood T, Machowicz R, Berliner N, et al. Recommendations for the management of hemophagocytic lymphohistiocytosis in adults. *Blood.* (2019) 133:2465–77. doi: 10.1182/blood.2018894618
8. Beutel G, Wiesner O, Eder M, Hafer C, Schneider AS, Kielstein JT, et al. Virus-associated hemophagocytic syndrome as a major contributor to death in patients with 2009 influenza A (H1N1) infection. *Crit Care.* (2011) 15:R80. doi: 10.1186/cc10073
9. Al-Ahmari A. Is secondary hemophagocytic lymphohistiocytosis behind the high fatality rate in Middle East respiratory syndrome corona virus? *J Appl Hematol.* (2015) 6:1–5. doi: 10.4103/1658-5127.155168
10. Ye Q, Wang B, Mao J. The pathogenesis and treatment of the 'Cytokine Storm' in COVID-19. *J Infect.* (2020) 80:607–13. doi: 10.1016/j.jinf.2020.03.037
11. Shive CL, Jiang W, Anthony DD, Lederman MM. Soluble CD14 is a nonspecific marker of monocyte activation. *AIDS.* (2015) 29:1263–5. doi: 10.1097/QAD.0000000000000735
12. Tippett E, Cheng WJ, Westhorpe C, Cameron PU, Brew BJ, Lewin SR, et al. Differential expression of CD163 on monocyte subsets in healthy and HIV-1 infected individuals. *PLoS One.* (2011) 6:e19968. doi: 10.1371/journal.pone.0019968
13. Sandler NG, Wand H, Roque A, Law M, Nason MC, Nixon DE, et al. Plasma levels of soluble CD14 independently predict mortality in HIV infection. *J Infect Dis.* (2011) 203:780–90. doi: 10.1093/infdis/jiq118
14. Lien E, Aukrust P, Sundan A, Muller F, Froland SS, Espevik T. Elevated levels of serum-soluble CD14 in human immunodeficiency virus type 1 (HIV-1) infection: correlation to disease progression and clinical events. *Blood.* (1998) 92:2084–92. doi: 10.1182/blood.V92.6.2084.418k26_2084_2092
15. Lederman MM, Calabrese L, Funderburg NT, Clagett B, Medvik K, Bonilla H, et al. Immunologic failure despite suppressive antiretroviral therapy is related to activation and turnover of memory CD4 cells. *J Infect Dis.* (2011) 204:1217–26. doi: 10.1093/infdis/jir507
16. Burdo TH, Soulas C, Orzechowski K, Button J, Krishnan A, Sugimoto C, et al. Increased monocyte turnover from bone marrow correlates with severity of SIV encephalitis and CD163 levels in plasma. *PLoS Pathog.* (2010) 6:e1000842. doi: 10.1371/journal.ppat.1000842
17. Mascia C, Pozzetto I, Kertusha B, Marocco R, Del Borgo C, Tieghi T, et al. Persistent high plasma levels of sCD163 and sCD14 in adult patients with measles virus infection. *PLoS One.* (2018) 13:e0198174. doi: 10.1371/journal.pone.0198174
18. Obeng JA, Amoruso A, Camaschella GL, Sola D, Brunelleschi S, Fresu LG. Modulation of human monocyte/macrophage activity by tocilizumab, abatacept and etanercept: an in vitro study. *Eur J Pharmacol.* (2016) 780:33–7. doi: 10.1016/j.ejphar.2016.03.028
19. Rinehart JJ, Balcerzak SP, Sagone AL, LoBuglio AF. Effects of corticosteroids on human monocyte function. *J Clin Invest.* (1974) 54:1337–43. doi: 10.1172/JCI107880
20. Christensen JE, Thomsen AR. Co-ordinating innate and adaptive immunity to viral infection: mobility is the key. *APMIS.* (2009) 117:338–55. doi: 10.1111/j.1600-0463.2009.02451.x
21. Burdo TH, Lo J, Abbara S, Wei J, DeLelys ME, Preffer F, et al. Soluble CD163, a novel marker of activated macrophages, is elevated and associated with noncalcified coronary plaque in HIV-infected patients. *J Infect Dis.* (2011) 204:1227–36. doi: 10.1093/infdis/jir520
22. Gong J, Dong H, Xia SQ, Huang YZ, Wang D, Zhao Y, et al. Correlation analysis between disease severity and inflammation-related parameters in patients with COVID-19 pneumonia. *medRxiv.* (2020). [Preprint]. doi: 10.1101/2020.02.25.20025643
23. Zhang C, Wu Z, Li JW, Zhao H, Wang GQ. The cytokine release syndrome (CRS) of severe COVID-19 and Interleukin-6 receptor (IL-6R) antagonist Tocilizumab may be the key to reduce the mortality. *Int J Antimicrob Agents.* (2020) 55:105954. doi: 10.1016/j.ijantimicag.2020.105954
24. He W, Kapate N, Shields CWT, Mitragotri S. Drug delivery to macrophages: a review of targeting drugs and drug carriers to macrophages for inflammatory diseases. *Adv Drug Deliv Rev.* (2019) (in press). doi: 10.1016/j.addr.2019.12.001
25. Sterner RM, Sakemura R, Cox MJ, Yang N, Khadka RH, Forsman CL, et al. GM-CSF inhibition reduces cytokine release syndrome and neuroinflammation but enhances CAR-T cell function in xenografts. *Blood.* (2019) 133:697–709. doi: 10.1182/blood-2018-10-881722
26. Gomez-Rial J, Martinon-Torres F. A strategy targeting monocyte-macrophage differentiation to avoid pulmonary complications in sars-cov2 infection. *Clin Immunol.* (2020) 216:108442. doi: 10.1016/j.clim.2020.108442
27. Gomez-Rial J, Rivero-Calle I, Salas A, Martinon-Torres F. Role of monocytes/macrophages in covid-19 pathogenesis: implications for therapy. *Infect. Drug Resist.* (2020) 13:2485–2493. doi: 10.2147/IDR.S258639

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular and Infection Microbiology

OPINION
published: 23 September 2020
doi: 10.3389/fcimb.2020.550571

The Possible Dual Role of the ACE2 Receptor in Asthma and Coronavirus (SARS-CoV2) Infection

Anna Cláudia Calvielli Castelo Branco[1,2], *Maria Notomi Sato*[2] and *Ricardo Wesley Alberca*[2]*

[1] Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil, [2] Laboratory of Dermatology and Immunodeficiencies, LIM-56, Department of Dermatology, School of Medicine and Institute of Tropical Medicine of São Paulo, University of São Paulo, São Paulo, Brazil

Keywords: SARS-CoV2, asthma, COVID 19, ACE2, inflammation

OPEN ACCESS

Edited by:
Clinton Mathias, Western New England University, United States

Reviewed by:
Binod Kumar, Loyola University Chicago, United States

***Correspondence:**
Ricardo Wesley Alberca
ricardowesley@gmail.com

Specialty section:
This article was submitted to Virus and Host, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: *14 April 2020*
Accepted: *26 August 2020*
Published: *23 September 2020*

Citation:
Branco ACCC, Sato MN and Alberca RW (2020) The Possible Dual Role of the ACE2 Receptor in Asthma and Coronavirus (SARS-CoV2) Infection. Front. Cell. Infect. Microbiol. 10:550571. doi: 10.3389/fcimb.2020.550571

A new virus belonging to the *coronaviridae* family has been identified and named severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) (Zhu et al., 2020). This virus can generate a severe respiratory disease named coronavirus disease-2019 (COVID-19). In November 2019, SARS-CoV-2 began to infect humans and cause high rates of respiratory disorders worldwide; accordingly, COVID-19 was declared a pandemic in March 2020 (Li J. Y. et al., 2020). COVID-19 has already affected millions of people and killed over 600,000 individuals worldwide (WHO, 2020).

Similar to severe acute respiratory syndrome coronavirus-1 (SARS-CoV-1), which was responsible for the 2002 pandemic, SARS-CoV-2 infection is initiated when its S-protein binds to the angiotensin-converting enzyme 2 (ACE2) receptor through which it gains entry into the host's cells (Kuba et al., 2005; Walls et al., 2020). The affinity of SARS-CoV-2 for the ACE2 receptor is 10 times higher than that of SARS-CoV-1 (Wrapp et al., 2020). This receptor is mainly expressed in the lungs and to a lesser degree in other organs, such as the heart, kidneys, and intestines (Bavishi et al., 2020), which could explain the increased prevalence of lung infection.

Many risk factors are associated with the course and severity of COVID-19, including older age, systemic arterial hypertension (SAH), pregnancy, and obesity (Alberca et al., 2020a,b). Although asthma is a debilitating pulmonary syndrome, initial reports have not identified asthmatic patients as having a higher risk for COVID-19 (Guan et al., 2020; Zhang et al., 2020). In this manuscript, we review the possible association between the SARS-CoV-2 entry receptor ACE2 and asthma.

ASTHMA

Asthma is a complex respiratory syndrome that affects ∼350 million people worldwide (Global Initiative for Asthma, 2018). This disease is generally defined by restricted airflow, airway inflammation, and airway hyperresponsiveness, resulting in symptoms such as shortness of breath, wheezing, and cough; moreover, if untreated, asthma can be lethal (Global Initiative for Asthma, 2018). Asthma mortality seems to be declining worldwide (Ebmeier et al., 2017), and it is estimated that in 2015, over 400,000 deaths occurred due to asthma complications (Soriano et al., 2017). Asthma is also associated with other comorbidities, including SAH (Ferguson et al., 2014) and pulmonary hypertension (Rosival, 1990), which are two established risk factors for COVID-19 (Zhang et al., 2020). Other characteristics associated with the worst asthma symptoms, such as obesity and old age, are also associated with poor COVID-19 prognoses (Schatz et al., 2014; Skloot et al., 2016; Płusa, 2017; Zhang et al., 2020).

Asthmatic patients can suffer from a progressive worsening of symptoms called asthma exacerbation, which necessitates treatment with systemic corticosteroids and eventually mechanical ventilation and intensive care (Dougherty and Fahy, 2009). A common concept in asthma is that viral infections can be associated with asthma exacerbation (Costa et al., 2014), and in respiratory

viral infections, asthma patients can upregulate a wide range of molecules expressed in the lungs; one of these molecules is ACE2 (Bai et al., 2015).

SARS-COV AND ACE2

The ACE2 receptor is crucial for COVID-19, as SARS-CoV-2 can only enter ACE2-expressing cells (Zhou et al., 2020). Nevertheless, ACE-2 expression is also important for the control of lung inflammation and damage upon viral infection (Imai et al., 2005; Yang et al., 2014; Zou et al., 2014).

During SARS-CoV-1 infection, the overexpression of ACE2 increases viral infection and replication rate (Li et al., 2003), and in animal models, infection with SARS-CoV-1 is ACE2-dependent (Kuba et al., 2005). However, in SARS-COV-2 infection, Chen et al. proposed a negative association linking ACE2 expression and COVID-19 fatality, as ACE2 expression is reduced in elderly and type II diabetic patients (Yoon et al., 2016; Chen et al., 2020).

Interestingly, children also express low levels of ACE2 in the lungs (Bunyavanich et al., 2020), and the death rate in this group has been described as low (Bialek et al., 2020). In addition to the lower ACE2 expression in the lungs of elderly patients (Wu and McGoogan, 2020), other characteristics, such as the presence of other comorbidities, immune senescence, or low-grade inflammation associated with aging (inflammaging), could influence COVID-19 outcomes (Franceschi and Campisi, 2014; Fuentes et al., 2017).

Another important finding is that men infected with SARS-CoV-2 have more severe disease and higher mortality than women (Sharma et al., 2020). The primary female sex hormone (estrogen), in addition to being able to directly influence immune responses, is able to upregulate the expression of ACE2 (Bukowska et al., 2017).

Recently, it has also been described that some structural variations in the ACE2 receptor can lead to differences in protein binding with SARS-CoV-2, helping to understand different infection profiles in humans and even cases of viral resistance (Hussain et al., 2020). ACE2 may also play a larger role in SARS-CoV-2 infection, participating in postinfection regulation of the immune response, cytokine secretion, and viral genome replication (He et al., 2020).

ASTHMA AND COVID-19

A report from Wuhan, China, identified a low number of asthmatic patients among COVID-19 patients (0.9%); however, asthma was not associated with greater COVID-19 severity or mortality (Li X. et al., 2020). Another study with 5,700 COVID-19 patients from New York City, USA, identified 479 patients with asthma (9%) (Richardson et al., 2020). In addition to this discrepancy in the incidence of asthma among COVID-19 patients, a recent study with 1,827 patients identified that mortality was similar in asthmatic and non-asthmatic COVID-19 patients (Wang et al., 2020).

Song et al. evaluated the prevalence of asthma and chronic obstructive pulmonary disease (COPD) in patients from a cohort of COVID-19 patients in China and found that 2.3% of the patients had asthma and 2.2% had COPD; none of the patients had asthma and COPD (Song et al., 2020). They verified that COPD patients had a higher risk of severe COVID-19 than asthmatic patients. In addition, the number of ACE2-positive cells in alveolar epithelial cells was lower in asthmatic patients and higher in COPD patients than that in patients without asthma or COPD (Song et al., 2020).

ASTHMA, CORONAVIRUSES, AND ACE2

Asthma is the most common chronic disease in children (Ferrante and La Grutta, 2018), and in the previous pandemic caused by SARS-CoV-1, asthmatic children infected with SARS-CoV-1 did not sustain an increase in asthma exacerbation (Van Bever et al., 2004). Moreover, a 2019 report indicated that the most common chronic condition in Middle East respiratory syndrome coronavirus (MERS-CoV) patients was asthma (van Kerkhove et al., 2019). In another report, two patients who died from MERS-CoV complications had chronic respiratory syndromes: one had COPD and one had asthma (Min et al., 2016). Therefore, similar viral infections do not present a clear picture of how SARS-CoV-2 infection progresses in asthmatic patients.

In a murine asthma model, ACE2 activation has been implicated in a reduction in airway inflammatory response (Dhawale et al., 2016). It is important to highlight that asthma can be divided into four different endotypes: T helper cell (Th)2 high/eosinophilic, Th17/neutrophilic, Th2/Th17/mixed inflammation, and paucigranulocytic (without an increase in polymorphous nuclear cells in the lungs) (Wenzel, 2013).

The asthma endotype is especially important, as cytokines can modify ACE expression. IL-17 can upregulate ACE2 expression (Song et al., 2020), whereas IL-4 and IL-13 can downregulate ACE2 expression (Kimura et al., 2020; Song et al., 2020).

Eosinophils may also play a larger role in COVID-19, as non-asthmatic patients with COVID-19 who present an absence of eosinophils in the first day of hospitalization have a worst prognose than non-asthmatic patients with eosinophils (Tanni et al., 2020). Another study suggested that eosinophil count in peripheral blood has prognostic value, as patients with a low number of eosinophils were more likely to exhibit shortness of breath and require longer hospitalization time (Xie et al., 2020). An increase in eosinophils is associated with COVID-19 improvement and hospital release (Liu et al., 2020; Xie et al., 2020). We hypothesize that different endotypes of asthma may modify ACE2 expression differently, thereby affecting COVID-19.

ACE2 expression in the lungs is also modulated in other respiratory diseases. In an experimental model of smoke-induced acute respiratory distress, a Th17/neutrophilic syndrome, ACE2 was upregulated (Wösten-Van Asperen et al., 2011). In addition, cytokine release from smoking-associated lung injury induces upregulation of ACE2 in the lungs (Leung et al., 2020). In summary, different endotypes of asthma and patients with multiple characteristics, such as smoking asthmatic patients or asthmatic patients with other morbidities, may also present a difference in lung ACE2 levels.

ASTHMA TREATMENT AND COVID-19

Approximately 50–70% of asthmatic patients have Th2 high/eosinophilic asthma (Peters et al., 2014; Seys et al., 2017). Th2 high/eosinophilic asthma can be treated with allergen-specific immunotherapy or symptomatic medication. Allergen-specific immunotherapy is a process that usually increases the circulation of regulatory IL-10-producing cells (Asamoah et al., 2017; Alberca-Custodio et al., 2020), which could help to curb the pro-inflammatory cytokine storm in COVID-19. Asthma medications, such as corticosteroids and long-acting beta agonists, reduce lung inflammation and provide symptomatic control (Asamoah et al., 2017). Recently, the usage of inhaled corticosteroids was also associated with lower expression of ACE2 in the sputum of asthmatic patients (Peters et al., 2020).

Dexamethasone, a long-acting glucocorticoid commonly used in the treatment of asthma exacerbation (Shefrin and Goldman, 2009; Cross et al., 2011), has recently shown positive results in COVID-19 (Recovery Collaborative Group et al., 2020). Dexamethasone treatment reduced mortality among COVID-19 patients receiving respiratory support (RS) but not among patients not receiving RS (Recovery Collaborative Group et al., 2020).

Other anti-asthma drugs (AADs), mainly cromolyn, fenoterol, montelukast, and reproterol, have been postulated to be of potential use in SARS-CoV-2 infection (Wu et al., 2020). These drugs could help reduce inflammation and improve lung function (Mombeini et al., 2012; Davino-Chiovatto et al., 2019). Therefore, both dexamethasone and AAD usage for the treatment of asthma could confer additional protection to asthmatic patients.

Immunobiological treatment, including the use of monoclonal antibodies targeting asthma-related molecules, such as IL-5 and IgE, has proven effective in reducing asthma symptoms (Samitas et al., 2015; Farne et al., 2017). To date, there is no report on the influence of anti-IL-5 on SARS-CoV-2 infection; therefore, the usage of this immunobiological agent during COVID-19 is contraindicated, as this type-2 cytokine could potentially counteract the type-1 cytokines released during infection (Vultaggio et al., 2020).

Interestingly, treatment with anti-IgE decreases endothelin-1 (Zietkowski et al., 2010), and the decrease in endothelin-1 upregulates the expression of ACE2 in bronchial epithelial cells (Zhang et al., 2013). On the other hand, a case of COVID-19 in a patient with severe asthma treated with the anti-IgE antibody did not provide evidence of asthma exacerbation or pneumonia (Lommatzsch et al., 2020). Hence, further studies with a larger cohort are necessary to better understand the role of this immunobiological treatment during COVID-19 and the corresponding influence on the ACE-2 receptor.

COVID-19 CYTOKINES, ACE2, AND ASTHMA

SARS-CoV-2 infection can generate a process called a cytokine storm, which is characterized by an increase in the levels of mainly type-1 cytokines, including IL-1, IL-8, IFN-γ, IP10, MCP1, and TNF, in the blood (Huang C. et al., 2020). The concentration of these cytokines can be a predictive factor in a patient's disease course (Huang C. et al., 2020). Investigations on the influence of the interaction between comorbidities, COVID-19 and cytokines on ACE2 expression are crucial for the development of new treatments for COVID-19 (Pagliaro and Penna, 2020). The upregulation of ACE2 is associated with an increase in the levels of IL-1, IL-10, IL-6, and IL-8 (He et al., 2020), which are important cytokines in the pathophysiology of COVID-19 (Guan et al., 2020; Zhang et al., 2020). IL-1 and IL-6 are likely involved in the development of fever, which is the most common COVID-19 symptom (Cartmell et al., 2000; Fabricio et al., 2006). IL-8, or CXCL8, is an important chemokine for the migration of neutrophils to the lungs in acute respiratory distress and the formation of neutrophil extracellular traps in COVID-19 (Wong et al., 2004; Gong et al., 2020; Middleton et al., 2020).

Although ACE2 plays a crucial role in SARS-CoV-2 viral infection, the use of ACE2 inhibitors may not be possible due to ACE2 being a protective factor in acute lung injury (Ye and Liu, 2020). Currently, no specific treatment or vaccine is available for SARS-CoV-2/COVID-19 (Huang L. et al., 2020). A preliminary study by Leng et al. showed that transplantation of seven patients from Beijing YouAn Hospital, China, with ACE2-negative mesenchymal stem cells (MSCs) was effective in improving the clinical outcomes of pneumonia, mainly due to immune modulation, with decreased TNF and increased IL-10 (Leng et al., 2020). Other reports have highlighted the usage of anti-TNF (Brito et al., 2020) and anti-IL-1β to regulate inflammation in COVID-19 patients (Cauchois et al., 2020).

IL-4 and IL-13, which are cytokines highly produced in Th2/eosinophilic asthma, can downregulate ACE2 expression in airway epithelial cells (Kimura et al., 2020; Song et al., 2020), whereas, TNF, IL-12, and IL-17A, which are cytokines highly produced in Th17/neutrophilic asthma and COPD (Alcorn et al., 2009), can upregulate ACE2 expression in *in vitro* BEAS-2B cells (Song et al.,

2020). In addition, circulating soluble angiotensin-converting enzyme 2 (sACE2) is upregulated in the blood of asthmatic patients (Ayada et al., 2015); hence, sACE2 could act as a competitive interceptor, limiting SARS-CoV-2 attachment to airway cell membranes (Batlle et al., 2020).

Further investigations are needed to better understand the role of ACE2 in asthmatic patients during SARS-CoV-2 infection, which would enable the development of better and more effective treatments for the COVID-19 pandemic while mitigating deaths in asthmatic individuals and the overall population.

AUTHOR CONTRIBUTIONS

AB and MS: write and review. RA: conception, write, and review. All authors contributed to the article and approved the submitted version.

FUNDING

RA has a post-doctorate fellowship of FAPESP (19/02679-7). Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES grant: 88887.503842/2020-00.

REFERENCES

Alberca, R. W., Oliveira de, M., Branco, A. C. C. C., Pereira, N. Z., and Sato, M. N. (2020b). Obesity as a risk factor for COVID-19: an overview. *Crit. Rev. Food Sci. Nutr.* 15, 1–15. doi: 10.1080/10408398.2020.1775546

Alberca, R. W., Pereira, N. Z., Oliveira, L. M. D. S., Gozzi-Silva, S. C., and Sato, M. N. (2020a). Pregnancy, viral infection, and COVID-19. *Front. Immunol.* 11:1672. doi: 10.3389/FIMMU.2020.01672

Alberca-Custodio, R. W., Faustino, L. D., Gomes, E., Nunes, F. P. B., de Siqueira, M. K., Labrada, A., et al. (2020). Allergen-specific immunotherapy with liposome containing CpG-ODN in murine model of asthma relies on MyD88 signaling in dendritic cells. *Front. Immunol.* 11:692. doi: 10.3389/fimmu.2020.00692

Alcorn, J. F., Crowe, C. R., and Kolls, J. K. (2009). TH17 cells in asthma and COPD. *Annu. Rev. Physiol.* 72, 495–516. doi: 10.1146/annurev-physiol-021909-135926

Asamoah, F., Kakourou, A., Dhami, S., Lau, S., Agache, I., Muraro, A., et al. (2017). Allergen immunotherapy for allergic asthma: a systematic overview of systematic reviews. *Clin. Transl. Allergy* 7:25. doi: 10.1186/s13601-017-0160-0

Ayada, C., Toru, Ü., Genç O., Sahin, S., Bulut Arik, Ö., and Acat, M. (2015). Evaluation of serum levels of renin angiotensin system components in asthmatic patients. *Erciyes Med. J.* 37, 87–90. doi: 10.5152/etd.2015.0027

Bai, J., Smock, S. L., Jackson, G. R., MacIsaac, K. D., Huang, Y., Mankus, C., et al. (2015). Phenotypic responses of differentiated asthmatic human airway epithelial cultures to rhinovirus. *PLoS ONE* 10:e01118286. doi: 10.1371/journal.pone.0118286

Batlle, D., Wysocki, J., and Satchell, K. (2020). Soluble angiotensin-converting enzyme 2: A potential approach for coronavirus infection therapy? *Clin. Sci.* 134, 543–545. doi: 10.1042/CS20200163

Bavishi, C., Maddox, T. M., and Messerli, F. H. (2020). Coronavirus disease 2019 (COVID-19) infection and renin angiotensin system blockers. *JAMA Cardiol.* 2020:1282. doi: 10.1001/jamacardio.2020

Bialek, S., Gierke, R., Hughes, M., McNamara, L. A., Pilishvili, T., and Skoff, T. (2020). Coronavirus disease 2019 in children — United States, february 12–april 2, 2020. *MMWR Morb. Mortal Wkly. Rep.* 69, 422–426. doi: 10.15585/mmwr.mm6914e4

Brito, C. A., Paiva, J. G., Pimentel, F. N., Guimarães, R. S., and Moreira, M. R. (2020). COVID-19 in patients with rheumatological diseases treated with anti-TNF. *Ann. Rheum. Dis.* doi: 10.1136/annrheumdis-2020-218171

Bukowska, A., Spiller, L., Wolke, C., Lendeckel, U., Weinert, S., Hoffmann, J., et al. (2017). Protective regulation of the ACE2/ACE gene expression by estrogen in human atrial tissue from elderly men. *Exp. Biol. Med.* 242, 1412–1423. doi: 10.1177/1535370217718808

Bunyavanich, S., Do, A., and Vicencio, A. (2020). Nasal gene expression of angiotensin-converting enzyme 2 in children and adults. *JAMA* 323:2427. doi: 10.1001/jama.2020.8707

Cartmell, T., Poole, S., Turnbull, A. V., Rothwell, N. J., and Luheshi, G. N. (2000). Circulating interleukin-6 mediates the febrile response to localised inflammation in rats. *J. Physiol.* 526, 653–661. doi: 10.1111/j.1469-7793.2000.00653.x

Cauchois, R., Koubi, M., Delarbre, D., Manet, C., Carvelli, J., Blasco, V. B., et al. (2020). Early IL-1 receptor blockade in severe inflammatory respiratory failure complicating COVID-19. *Proc. Natl. Acad. Sci. U.S.A.* 117, 18951–18953. doi: 10.1073/pnas.2009017117

Chen, J., Jiang, Q., Xia, X., Liu, K., Yu, Z., Tao, W., et al. (2020). Individual variation of the SARS-CoV-2 receptor ACE2 gene expression and regulation. *Aging Cell* 19:e13168. doi: 10.1111/acel.13168

Costa, L. D. C., Costa, P. S., and Camargos, P. A. M. (2014). Exacerbation of asthma and airway infection: Is the virus the villain? *J. Pediatr.* 90, 542–555. doi: 10.1016/j.jped.2014.07.001

Cross, K. P., Paul, R. I., and Goldman, R. D. (2011). Single-dose dexamethasone for mild-to-moderate asthma exacerbations: effective, easy, and acceptable. *Can. Fam. Phys.* 57, 1134–1136.

Davino-Chiovatto, J. E., Oliveira-Junior, M. C., MacKenzie, B., Santos-Dias, A., Almeida-Oliveira, A. R., Aquino-Junior, J. C. J., et al. (2019). Montelukast, leukotriene inhibitor, reduces LPS-induced acute lung inflammation and human neutrophil activation. *Arch. Bronconeumol.* 55, 573–580. doi: 10.1016/j.arbr.2019.10.002

Dhawale, V. S., Amara, V. R., Karpe, P. A., Malek, V., Patel, D., and Tikoo, K. (2016). Activation of angiotensin-converting enzyme 2 (ACE2) attenuates allergic airway inflammation in rat asthma model. *Toxicol. Appl. Pharmacol.* 306, 17–26. doi: 10.1016/j.taap.2016.06.026

Dougherty, R. H., and Fahy, J. V. (2009). Acute exacerbations of asthma: epidemiology, biology and the exacerbation-prone phenotype. *Clin. Exp. Allergy* 39, 193–202. doi: 10.1111/j.1365-2222.2008.03157.x

Ebmeier, S., Thayabaran, D., Braithwaite, I., Bénamara, C., Weatherall, M., and Beasley, R. (2017). Trends in international asthma mortality: analysis of data from the WHO mortality database from 46 countries (1993–2012). *Lancet* 390, 935–945. doi: 10.1016/S0140-6736(17)31448-4

Fabricio, A. S. C., Tringali, G., Pozzoli, G., Melo, M. C., Vercesi, J. A., Souza, G. E. P., et al. (2006). Interleukin-1 mediates endothelin-1-induced fever and prostaglandin production in the preoptic area of rats. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 290, R1515–R1523. doi: 10.1152/ajpregu.00604.2005

Farne, H. A., Wilson, A., Powell, C., Bax, L., and Milan, S. J. (2017). Anti-IL5 therapies for asthma. *Cochrane Database Syst. Rev.* 9:CD010834. doi: 10.1002/14651858.CD010834.pub3

Ferguson, S., Teodorescu, M. C., Gangnon, R. E., Peterson, A. G., Consens, F. B., Chervin, R. D., et al. (2014). Factors associated with systemic hypertension in asthma. *Lung* 192, 675–683. doi: 10.1007/s00408-014-9600-y

Ferrante, G., and La Grutta, S. (2018). The burden of pediatric asthma. *Front. Pediatr.* 6:186. doi: 10.3389/fped.2018.00186

Franceschi, C., and Campisi, J. (2014). Chronic inflammation (Inflammaging) and its potential contribution to age-associated diseases. *J. Gerontol. Ser A Biol. Sci. Med. Sci.* 69(Suppl. 1), S4–9. doi: 10.1093/gerona/glu057

Fuentes, E., Fuentes, M., Alarcón, M., and Palomo, I. (2017). Immune system dysfunction in the elderly. *An. Acad. Bras. Cienc.* 89, 285–299. doi: 10.1590/0001-3765201720160487

Global Initiative for Asthma (2018). *Global Strategy for Asthma Management and* Prevention. Vancouver: GINA. Available online at: www.ginasthma.org (accessed September 08, 2020)

Gong, J., Dong, H., Xia, S. Q., Huang, Y. Z., Wang, D., Zhao, Y., et al. (2020). Correlation analysis between disease severity and inflammation-related parameters in patients with COVID-19 pneumonia. *Cold Spring Harbor Lab.* doi: 10.1101/2020.02.25.20025643

Guan, W., Ni, Z., Hu, Y., Liang, W., Ou, C., He, J., et al. (2020). Clinical characteristics of coronavirus disease 2019 in China.

N. Engl. J. Med. 382, 1708–1720. doi: 10.1056/nejmoa2002032

He, X., Zhang, L., Ran, Q., Xiong, A., Wang, J., Wu, D., et al. (2020). Integrative bioinformatics analysis provides insight into the molecular mechanisms of 2019-nCoV. *medRxiv*. doi: 10.1101/2020.02.03.20020206

Huang, C., Wang, Y., Li, X., Ren, L., Zhao, J., Hu, Y., et al. (2020). Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet* 395, 497–506. doi: 10.1016/S0140-6736(20)30183-5

Huang, L., Chen, Y., Xiao, J., Luo, W., Li, F., Wang, Y., et al. (2020). Progress in the Research and Development of Anti-COVID-19 Drugs. *Front. Pub. Heal* 8:365. doi: 10.3389/fpubh.2020.00365

Hussain, M., Jabeen, N., Raza, F., Shabbir, S., Baig, A. A., Amanullah, A., et al. (2020). Structural variations in human ACE2 may influence its binding with SARS-CoV-2 spike protein. *J. Med. Virol.* 92, 1580–1586. doi: 10.1002/jmv.25832

Imai, Y., Kuba, K., Rao, S., Huan, Y., Guo, F., Guan, B., et al. (2005). Angiotensin-converting enzyme 2 protects from severe acute lung failure. *Nature* 436, 112–116. doi: 10.1038/nature03712

Kimura, H., Francisco, D., Conway, M., Martinez, F. D., Vercelli, D., Polverino, F., et al. (2020). Type 2 inflammation modulates ACE2 and TMPRSS2 in airway epithelial cells. *J. Allergy Clin. Immunol.* 146, 80–88.e8. doi: 10.1016/j.jaci.2020.05.004

Kuba, K., Imai, Y., Rao, S., Gao, H., Guo, F., Guan, B., et al. (2005). A crucial role of angiotensin converting enzyme 2 (ACE2) in SARS coronavirus-induced lung injury. *Nat. Med.* 11, 875–879. doi: 10.1038/nm1267

Leng, Z., Zhu, R., Hou, W., Feng, Y., Yang, Y., Han, Q., et al. (2020). Transplantation of ACE2- mesenchymal stem cells improves the outcome of patients with COVID-19 pneumonia. *Aging Dis.* 11:216. doi: 10.14336/ad.2020.0228

Leung, J. M., Yang, C. X., Tam, A., Shaipanich, T., Hackett, T. L., Singhera, G. K., et al. (2020). ACE-2 expression in the small airway epithelia of smokers and COPD patients: implications for COVID-19. *Eur. Respir. J.* 55:2000688. doi: 10.1183/13993003.00688-2020

Li, J. Y., You, Z., Wang, Q., Zhou, Z. J., Qiu, Y., Luo, R., et al. (2020). The epidemic of 2019-novel-coronavirus (2019-nCoV) pneumonia and insights for emerging infectious diseases in the future. *Microbes Infect.* 22, 80–85. doi: 10.1016/j.micinf.2020.02.002

Li, W., Moore, M. J., Vasllieva, N., Sui, J., Wong, S. K., Berne, M. A., et al. (2003). Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. *Nature* 426, 450–454. doi: 10.1038/nature02145

Li, X., Xu, S., Yu, M., Wang, K., Tao, Y., Zhou, Y., et al. (2020). Risk factors for severity and mortality in adult COVID-19 inpatients in Wuhan. *J. Allergy Clin. Immunol.* 146, 110–118. doi: 10.1016/j.jaci.2020.04.006

Liu, F., Xu, A., Zhang, Y., Xuan, W., Yan, T., Pan, K., et al. (2020). Patients of COVID-19 may benefit from sustained lopinavir-combined regimen and the increase of eosinophil may predict the outcome of COVID-19 progression. *Int. J. Infect Dis.* 95, 183–191. doi: 10.1016/j.ijid.2020.03.013

Lommatzsch, M., Stoll, P., and Virchow, J. C. (2020). COVID-19 in a patient with severe asthma treated with Omalizumab. *Allergy*. doi: 10.1111/all.14456

Middleton, E. A., He, X.-Y., Denorme, F., Campbell, R. A., Ng, D., Salvatore, S. P., et al. (2020). Neutrophil extracellular traps (NETs) contribute to immunothrombosis in COVID-19 acute respiratory distress syndrome. *Blood* 136, 1169–1179. doi: 10.1182/blood.2020007008

Min, C.-K., Cheon, S., Ha, N.-Y., Sohn, K. M., Kim, Y., Aigerim, A., et al. (2016). Comparative and kinetic analysis of viral shedding and immunological responses in MERS patients representing a broad spectrum of disease severity. *Sci. Rep.* 6:25359. doi: 10.1038/srep25359

Mombeini, T., Zahedpoure-Anaraki, M. R., and Dehpour, A. R. (2012). Effects of sodium cromoglycate on Iranian asthmatic subjects without exposure to any bronchoconstrictor agent. *Iran J. Pharm. Res.* 11, 549–557. doi: 10.22037/ijpr.2012.1149

Pagliaro, P., and Penna, C. (2020). ACE/ACE2 ratio: a key also in 2019 coronavirus disease (Covid-19)? *Front. Med.* 7:335. doi: 10.3389/fmed.2020.00335

Peters, M. C., Mekonnen, Z. K., Yuan, S., Bhakta, N. R., Woodruff, P. G., and Fahy, J. V. (2014). Measures of gene expression in sputum cells can identify T H2-high and TH2-low subtypes of asthma. *J. Allergy Clin. Immunol.* 133, 388–394. doi: 10.1016/j.jaci.2013.07.036

Peters, M. C., Sajuthi, S., Deford, P., Christenson, S., Rios, C. L., Montgomery, M. T., et al. (2020). COVID-19-related genes in sputum cells in asthma: relationship to demographic features and corticosteroids. *Am. J. Respir. Crit. Care Med*. 202, 83–90. doi: 10.1164/rccm.202003-0821OC

Płusa, T. (2017). Obesity and asthma. *Int. Rev. Allergol. Clin. Immunol. Fam. Med.* 141, 1169–1179. doi: 10.2174/187221409789104773

Recovery Collaborative Group, Horby, P., Lim, W. S., Emberson, J. R., Mafham, M., Bell, J. L., et al. (2020). Dexamethasone in hospitalized patients with Covid-19 - preliminary report. *N. Engl. J. Med.* doi: 10.1056/NEJMoa2021436

Richardson, S., Hirsch, J. S., Narasimhan, M., Crawford, J. M., McGinn, T., Davidson, K. W., et al. (2020). Presenting characteristics, comorbidities, and outcomes among 5700 patients hospitalized with COVID-19 in the New York City Area. *JAMA* 323, 2052–2059. doi: 10.1001/jama.2020.6775

Rosival, V. (1990). The role of pulmonary hypertension in the pathogenesis of bronchial asthma. *Med. Hypotheses* 33, 7–9. doi: 10.1016/0306-9877(90)90076-q

Samitas, K., Delimpoura, V., Zervas, E., and Gaga, M. (2015). Anti-IgE treatment, airway inflammation and remodelling in severe allergic asthma: current knowledge and future perspectives. *Eur. Respir. Rev.* 24, 594–601. doi: 10.1183/16000617.00001715

Schatz, M., Hsu, J. W. Y., Zeiger, R. S., Chen, W., Dorenbaum, A., Chipps, B. E., et al. (2014). Phenotypes determined by cluster analysis in severe or difficult-to-treat asthma. *J. Allergy Clin. Immunol.* 133, 1549–1556. doi: 10.1016/j.jaci.2013.10.006

Seys, S. F., Scheers, H., Van den Brande, P., Marijsse, G., Dilissen, E., Van Den Bergh, A., et al. (2017). Cluster analysis of sputum cytokine-high profiles reveals diversity in T(h)2-high asthma patients. *Respir. Res.* 18:39. doi: 10.1186/s12931-017-0524-y

Sharma, G., Volgman, A. S., and Michos, E. D. (2020). Sex differences in mortality from COVID-19 pandemic. *JACC Case Rep.* 2, 1407–1410. doi: 10.1016/j.jaccas.2020.04.027

Shefrin, A. E., and Goldman, R. D. (2009). Use of dexamethasone and prednisone in acute asthma exacerbations in pediatric patients. *Can. Fam. Phys.* 55, 704–706.

Skloot, G. S., Busse, P. J., Braman, S. S., Kovacs, E. J., Dixon, A. E., Vaz Fragoso, C. A., et al. (2016). An official American thoracic society workshop report: evaluation and management of asthma in the elderly. *Ann. Am. Thorac. Soc.* 13, 2064–2077. doi: 10.1513/AnnalsATS.201608-658ST

Song, J., Zeng, M., Wang, H., Qin, C., Hou, H., Sun, Z., et al. (2020). Distinct effects of asthma and COPD comorbidity on disease expression and outcome in patients with COVID-19. *Allergy* 27:14517. doi: 10.1111/all.14517

Soriano, J. B., Abajobir, A. A., Abate, K. H., Abera, S. F., Agrawal, A., Ahmed, M. B., et al. (2017). Global, regional, and national deaths, prevalence, disability-adjusted life years, and years lived with disability for chronic obstructive pulmonary disease and asthma, 1990–2015: a systematic analysis for the global burden of disease study 2015. *Lancet Respir. Med.* 5, 691–706. doi: 10.1016/S2213-2600(17)30293-X

Tanni, F., Akker, E., Zaman, M. M., Figueroa, N., Tharian, B., and Hupart, K. H. (2020). Eosinopenia and COVID-19. *J. Am. Osteopath. Assoc.* 120, 504–508. doi: 10.7556/jaoa.2020.091

Van Bever, H. P., Chng, S. Y., and Goh, D. Y. (2004). Childhood severe acute respiratory syndrome, coronavirus infections and asthma. *Pediatr. Allergy Immunol.* 15, 206–209. doi: 10.1111/j.1399-3038.2004.00137.x

van Kerkhove, M. D., Aswad, S., Assiri, A., Perera, R. A. P. M., Peiris, M., El Bushra, H. E., et al. (2019). Transmissibility of MERS-CoV infection in closed setting, Riyadh, Saudi Arabia, 2015. *Emerg. Infect. Dis.* 25, 1802–1809. doi: 10.3201/eid2510.190130

Vultaggio, A., Agache, I., Akdis, C. A., Akdis, M., Bavbek, S., Bossios, A., et al. (2020). Considerations on biologicals for patients with allergic disease in times of the COVID-19 pandemic: an EAACI statement. *Allergy*. doi: 10.1111/all.14407

Walls, A. C., Park, Y. J., Tortorici, M. A., Wall, A., McGuire, A. T., and Veesler, D. (2020). Structure, function, and antigenicity of the SARS-CoV-2 spike glycoprotein. *Cell* 181, 281–292.e6. doi: 10.1016/j.cell.2020.02.058

Wang, L., Foer, D., Bates, D. W., Boyce, J. A., and Zhou, L. (2020). Risk factors for hospitalization, intensive care and mortality among patients with asthma and COVID-19. *J. Allergy Clin. Immunol.* doi: 10.1016/j.jaci.2020.07.018

Wenzel, S. E. (2013). Complex phenotypes in asthma: current definitions. *Pulm. Pharmacol. Ther.* 26, 710–715. doi: 10.1016/j.pupt.2013.07.003

WHO (2020). *Coronavirus Disease (COVID-19) Dashboard | WHO Coronavirus Disease (COVID-19) Dashboard*. Available online at: https://covid19.who.int/ (accessed July 30, 2020).

Wong, C. K., Lam, C. W. K., Wu, A. K. L., Ip, W. K., Lee, N. L. S., Chan, I. H. S., et al. (2004). Plasma inflammatory cytokines and chemokines in severe acute respiratory syndrome. *Clin. Exp. Immunol.* 136, 95–103. doi: 10.1111/j.1365-2249.2004.02415.x

Wösten-Van Asperen, R. M., Lutter, R., Specht, P. A., Moll, G. N., Van Woensel, J. B., Van Der Loos, C. M., et al. (2011). Acute respiratory distress syndrome leads to reduced ratio of ACE/ACE2 activities and is prevented by angiotensin-(1-7) or an angiotensin II receptor antagonist. *J. Pathol.* 225, 618–627. doi: 10.1002/path.2987

Wrapp, D., Wang, N., Corbett, K. S., Goldsmith, J. A., Hsieh, C. L., Abiona, O., et al. (2020). Cryo-EM structure of the 2019-nCoV spike in the prefusion conformation. *Science* 367, 1260–1263. doi: 10.1126/science.aax0902

Wu, C., Liu, Y., Yang, Y., Zhang, P., Zhong, W., Wang, Y., et al. (2020). Analysis of therapeutic targets for SARS-CoV-2 and discovery of potential drugs by computational methods. *Acta Pharm. Sin B* 10, 766–788. doi: 10.1016/j.apsb.2020.02.008

Wu, Z., and McGoogan, J. M. (2020). Characteristics of and important lessons from the coronavirus disease 2019 (COVID-19) outbreak in China. *JAMA*. 323, 1239–1242. doi: 10.1001/jama.2020.2648

Xie, G., Ding, F., Han, L., Yin, D., Lu, H., and Zhang, M. (2020). The role of peripheral blood eosinophil counts in COVID-19 patients. *Allergy*. 1–12. doi: 10.1111/all.14465

Yang, P., Gu, H., Zhao, Z., Wang, W., Cao, B., Lai, C., et al. (2014). Angiotensin-converting enzyme 2 (ACE2) mediates influenza H7N9 virus-induced acute lung injury. *Sci. Rep.* 4:7027. doi: 10.1038/srep07027

Ye, R., and Liu, Z. (2020). ACE2 exhibits protective effects against LPS-induced acute lung injury in mice by inhibiting the LPS-TLR4 pathway. *Exp. Mol. Pathol.* 113:104350. doi: 10.1016/j.yexmp.2019.104350

Yoon, H. E., Kim, E. N., Kim, M. Y., Lim, J. H., Jang, I. A., Ban, T. H., et al. (2016). Age-associated changes in the vascular renin-angiotensin system in mice. *Oxid. Med. Cell Longev.* 2016:6731093. doi: 10.1155/2016/6731093

Zhang, H., Li, Y., Zeng, Y., Wu, R., and Ou, J. (2013). Endothelin-1 downregulates angiotensin-converting enzyme-2 expression in human bronchial epithelial cells. *Pharmacology* 91, 297–304. doi: 10.1159/000350395

Zhang, J., Dong, X., Cao, Y., Yuan, Y., Yang, Y., Yan, Y., et al. (2020). Clinical characteristics of 140 patients infected with SARS-CoV-2 in Wuhan, China. *Allergy Eur. J. Allergy Clin. Immunol.* 75, 1730–1741. doi: 10.1111/all.14238

Zhou, P., Yang, X., Lou, Wang X. G., Hu, B., Zhang, L., Zhang, W., et al. (2020). A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature* 579, 270–273. doi: 10.1038/s41586-020-2012-7

Zhu, N., Zhang, D., Wang, W., Li, X., Yang, B., Song, J., et al. (2020). A novel coronavirus from patients with pneumonia in China, 2019. *N. Engl. J. Med.* 8, 727–733. doi: 10.1056/NEJMoa2001017

Zietkowski, Z., Skiepko, R., Tomasiak-Lozowska, M. M., and Bodzenta-Lukaszyk, A. (2010). Anti-IgE therapy with omalizumab decreases endothelin-1 in exhaled breath condensate of patients with severe persistent allergic asthma. *Respiration* 80, 534–542. doi: 10.1159/000317137

Zou, Z., Yan, Y., Shu, Y., Gao, R., Sun, Y., Li, X., et al. (2014). Angiotensin-converting enzyme 2 protects from lethal avian influenza A H5N1 infections. *Nat. Commun.* 5:3594. doi: 10.1038/ncomms4594

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cell and Developmental Biology

REVIEW
published: 24 September 2020
doi: 10.3389/fcell.2020.547314

How to Understand "Herd Immunity" in COVID-19 Pandemic

Yuanqing Xia[1,2†], Lumin Zhong[3†], Jingcong Tan[1†], Zhiruo Zhang[1], Jiajun Lyu[1], Yiting Chen[1], Anda Zhao[4], Lili Huang[1], Zichong Long[1], Ning-Ning Liu[1], Hui Wang[1*] and Shenghui Li[1,5*]*

[1] School of Public Health, Shanghai Jiao Tong University School of Medicine, Shanghai, China, [2] International Peace Maternity & Child Health Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China, [3] Department of Epidemiology and Public Health, University College London, London, United Kingdom, [4] Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China, [5] The Ministry of Education of the People's Republic of China (MOE)-Shanghai Key Laboratory of Childre's Environmental Health, Shanghai Jiao Tong University School of Medicine, Shanghai, China

The COVID-19 pandemic has been a global threat. Through rapid and effective surveillance and control, the newly confirmed patients have been fluctuated at a very low level and imported case explained most of them through March, 2020 to the present, indicating China's response has achieved a stage victory. By contrast, the epidemic of COVID-19 in other countries out of China is bursting. Different countries are adopting varied response strategy in terms of their public health system to prevent the spread. Herd immunity has been a hot topic since the outbreak of COVID-19 pandemic. Can it be a possible strategy to combat COVID-19? To fully interpret the knowledge regarding the term upon the background of COVID-19-related health crisis, we aim to systematically review the definition, describe the effective measures of acquiring herd immunity, and discuss its feasibility in COVID-19 prevention. Findings from this review would promote and strengthen the international cooperation and joint efforts when confronting with COVID-19.

Keywords: COVID-19, SARS-CoV-2, herd immunity, outbreak, pandemic

OPEN ACCESS

Edited by:
Tatsuo Shioda,
Osaka University, Japan

Reviewed by:
Dong-Joo (Ellen) Cheon,
Albany Medical College, United States
Emi E. Nakayama,
Osaka University, Japan

***Correspondence:**
Ning-Ning Liu
liuningning@shsmu.edu.cn;
liuningning@sibcb.ac.cn
Hui Wang
huiwang@shsmu.edu.cn
Shenghui Li
lsh9907@163.com

†These authors share first authorship

Specialty section:
This article was submitted to Molecular Medicine, a section of the journal Frontiers in Cell and Developmental Biology

Received: *31 March 2020*
Accepted: *02 September 2020*
Published: *24 September 2020*

Citation:
Xia Y, Zhong L, Tan J, Zhang Z, Lyu J, Chen Y, Zhao A, Huang L, Long Z, Liu N-N, Wang H and Li S (2020) How to Understand "Herd Immunity" in COVID-19 Pandemic. Front. Cell Dev. Biol. 8:547314. doi: 10.3389/fcell.2020.547314

INTRODUCTION

On March 11st, 2020, the world health organization (WHO) declared Coronavirus Disease 2019 (COVID-19) as a global pandemic. By 10am on August 30th, 217 countries or regions had reported confirmed cases, with a total of more than 25,070,000 cases (World Health Organization, 2020b). In early March, British Prime Minister Boris Johnson unveiled UK's plan to tackle the COVID-19 outbreak through four phases – Contain, Delay, Research and Mitigate. On March 12nd, the Prime Minister announced that the country had switched from the "Contain" to "Delay" phase. On March 13rd, Sir Patrick Vallance, the Government's chief scientific adviser, mentioned "herd immunity" and pointed out that passively waiting for "herd immunity" would lead to 60% of the population infected with COVID-19. Ever since then, "herd immunity" was top searched through the internet and remains to be a hot issue in debate. Even in April, Sweden's chief epidemiologist, Dr. Anders Tegnell, said Sweden was tackling the COVID-19 outbreak through "herd immunity." At approximately the same time, the United States and Australia were also under public scrutiny over whether the two countries were adopting "herd immunity" strategy. The term "herd immunity" has never been noticed by the general public before and this is unprecedented that it comes into our sight. Here, we choose China, the United Kingdom, Sweden, the United States,

and Australia as our settings, illustrating the theoretical basis of "herd immunity," and discuss its feasibility in the fight of curtaining COVID-19 outbreak.

WHAT IS "HERD IMMUNITY"?

"Herd immunity," as a concept in immunology, is used to describe the resistance to the spread of a contagious disease within a population or herd. The concept was first proposed in Topley and Wilson (1923) in their publication named the spread of bacterial infection: The problem of "herd immunity." Herd immunity only exists when a sufficiently high proportion of the population generate immunity against the foreign pathogen so that the probability of transmission between infected and susceptible individuals is reduced (Smith, 2019). In other words, it is becoming difficult for contagious disease to spread between individuals if herd immunity exists as the chain of transmission is broken and the susceptible individuals are protected from infection.

In 1933, Dr. Arthur W. Hedrich, health official of Chicago, Illinois, observed the phenomenon that the measles outbreak was prevented after 68% of children were infected between 1900 and 1930 in Boston, Massachusetts (Fine, 1993). The number of cases were kept to a low level after the measles vaccine was legalized in 1964 and the second dose was inoculated till the late 1980s (**Figure 1**; CDC, 1993; McNabb et al., 2007).

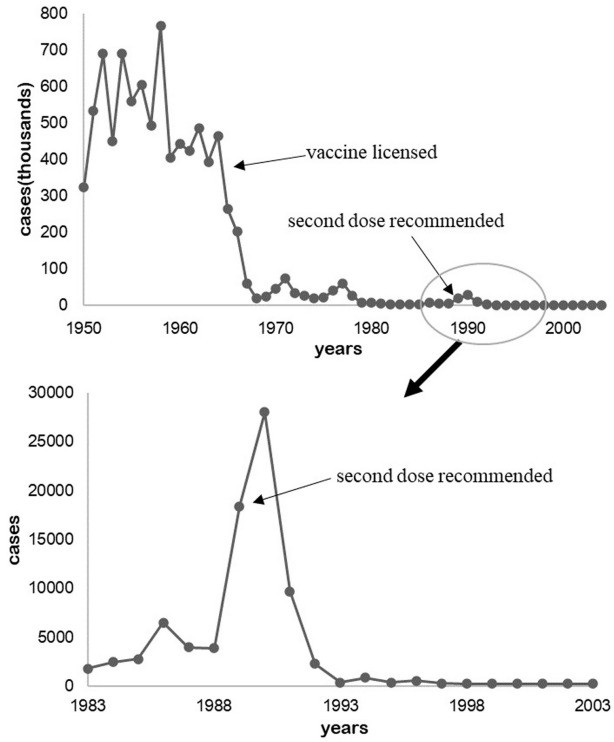


FIGURE 1 | Measles cases in the United States from 1950 to 2004.

HOW TO ACHIEVE HERD IMMUNITY?

Herd immunity is based on individual immunity which refers to a physiological function that the body's immune system recognizes and differentiates its own and alien substances and eliminates the antigenic substances (such as bacteria and viruses) through immune response to maintain health. It may be built up by confronting a disease or infection in the past and recovering from it. Immunity can also be induced by vaccination. Herd immunity is usually achieved by vaccination (e.g., smallpox vaccine) or by lots of people being infected with the contagious disease (e.g., influenza).

COVID-19 AND HERD IMMUNITY

The History of Herd Immunity

There are many examples in human history of blocking or even eliminating infectious diseases through herd immunity (Fine et al., 2011). Smallpox is considered to be among the most deadly infectious diseases human are generally prone to be infected. Its spread in populations initiated for thousands of years from ancient times to the recent human history (Theves et al., 2016). In 1979, smallpox was officially declared eradicated based on herd immunity achieved by intensive vaccination campaigns (Lane, 2006). Similarly, rinderpest, a highly contagious disease, was eradicated in 2011 through herd immunity in animals (Tounkara and Nwankpa, 2017). Other ubiquitous diseases such as measles, rubella, pertussis are not eradicated yet, herd immunity is maintained by keeping the proportion immune above some threshold to protect susceptible individuals (Black, 1982; Assaad, 1983; Fine et al., 2011). Up to now the number of cases were kept in a low level (Adams et al., 2017). In 1988, the incidence of measles in the United States fell to 1.3 cases per million following the introduction of measles vaccines by initiating two measles elimination efforts, and reemergence of indigenous transmission in the United States finally disappeared since 2000 (Katz and Hinman, 2004; Phadke et al., 2016) .After the whole-cell pertussis vaccines were widely used into routine childhood immunization in the mid- 1940s, there was a markable reduction in the pertussis incidence, from 150,000 to 260,000 cases to a nadir of just 1010 cases in 1976 annually (CDC, 1922-2018; Phadke et al., 2016).

Possible Outcomes After Herd Immunity

Since there's no approved vaccine for COVID-19 yet, the herd immunity cannot be achieved by vaccination. If herd immunity is derived from natural infection, what is the proportion of a population that need to be immunized in order to achieve the effect of protection? We can estimate this ratio based on the basic infection number (R_0, the expected average number of additional cases that one case will generate over the course of its infectious period in an otherwise uninfected and generally susceptible population) of COVID-19. The R_0s of some common vaccine-preventable contagious diseases are shown in **Table 1** (Fine et al., 2018). Based on the formula of herd immunity threshold (threshold = $1–1/R_0$) (Fine et al., 2011), and the R_0

TABLE 1 | R_0 and threshold value of herd immunity of common vaccine-preventable contagious diseases.

Disease	Route of transmission	R_0	Herd immunity threshold
Diphtheria	Saliva	6–7	83–85%
Measles	Air borne	12–18	92–94%
Mumps	Droplet spread	4–7	75–86%
Pertussis	Droplet spread	12–17	80–94%
Poliomyelitis	Fecal-oral transmission	5–7	50–95%
Rubella	Droplet spread	5–7	83–85%
Smallpox	Contact transmission	6–7	80–85%

of COVID-19 being 2.27 (Zhang et al., 2020), only when about 56% of population get specific immunity to SARS-CoV-2, then transmission-blocking can be achieved with herd immunity. Herd immunity in measles suggests the whole population is protected from emerging infections when 90% or more are immunized, whether by vaccination or recovery from natural infection. However, in the case of COVID-19, there are two outcomes when people get naturally infected – recovery and death. It is unclear whether those who are cured are free of the virus and exempted from the contagious, which indicates that the percentage of infected people would be more than 60–70%.

According to an epidemiological analysis of COVID-19 in China, COVID-19 can cause about 15% severe cases and a 2% death rate (World Health Organization., 2020a; Zhang et al., 2020). Particularly, the projections above were based on the existing data in China where the overall isolation and the centralized allocation of medical resources of the whole country are adopted. Without effective medical resources and isolation interventions, natural infection may result in a more severe mortality rate.

What will be the cost of the government's "herd immunity" or "mitigate" strategy? A simulation study about the pandemic trend by epidemiological model found that an estimated number of 510,000 British people will die if nothing is carried out, and about 250,000 British people would also die if mitigation measures were maximized (Ferguson et al., 2020). The study predicted that the peak in mortality would occur after 3 months and, given the estimated R_0 of 2.4, 81% of United Kingdom and United States populations would be infected.

Herd Immunity Lessons From Other Viruses

The length of duration of herd immunity was challenged by immune senescence, and the breadth of duration was challenged by antigenic diversity of a pathogen (Mallory et al., 2018). Over time, the progressive loss of responsiveness to a pathogen and the decreased antibody titer or cellular responses would result in loss of immunity. In the early 21th century, measles infections peaked in Chinese middle-aged adults after re-introduction of the wild-type virus, who had been immunized by early-age vaccination and then boosted with attenuated virus after a time interval (He et al., 2013). And this phenomenon also occurred in South Korea (Kang et al., 2017).

Generally, a viral species especially RNA viruses, consists of multiple antigenically distinct variants resulted from antigenic drift, antigenic shift, and recombination (Pica and Palese, 2013; Payne, 2017). However, most RNA polymerases lack a proof-reading function to solve it. It poses challenging obstacles in eliciting broad immunity through vaccination with a single serotype of attenuated virus, as evidenced by Norovirus (Debbink et al., 2014), dengue (Midgley et al., 2011), and influenza (Wu et al., 2017). Moreover, vaccination with a single serotype may increase the severity of a secondary infection, which ever occurred in Dengue virus with four serotypes (de Alwis et al., 2014). Similarly, vaccination with the bivalent HPV vaccine caused decline in the prevalence of HPV types 16 and 18 and cross-protection against non-vaccine types HPV 31, 33, and 45, but increased prevalence of non-vaccine, non–cross-protective HPV types (Brisson et al., 2016; Cameron et al., 2016; Ribeiro et al., 2020).

Being a Threat to the World

We are now living in an era of "the global village" with constant flux of large populations. There will be an "Immunity gap" if the majority of people gain antiviral immunity to SARS-CoV-2 in particular countries by natural infection but not in the other countries. Once the solid growth of economy is restored and traffic controls are lifted, there might be large-scale international transmissions with unsatisfactory outcomes. For example, in the early 16th century, the smallpox virus ever killed 3 million Indians who had never been exposed to it after it was introduced to America by European colonists who had been immunized against the smallpox virus (Eyler, 2003).

Besides, taking measles as another example, measles epidemics continues to occur even when the measles vaccine is widespread (Smiianov et al., 2019). In 2008, an unvaccinated 7-year-old boy contracted measles and infected 11 children, after returning home from a family vacation in Switzerland to San Diego, a city with a 95% measles vaccination rate (Sugerman et al., 2010). That proportion, by the concept of herd immunity, should be enough to keep measles at bay and protect those unvaccinated. This outbreak is mainly due to failure to vaccinate and importation of cases (Haralambieva et al., 2019). In fact, although the average vaccination rate may be high across the county, it varied locally. Rates in some neighborhoods may be far below the necessary threshold to achieve herd immunity (Peeples, 2019). Various studies have estimated that 2–10% of individuals vaccinated against measles may not develop immunity, allowing a gradual accumulation of susceptible individuals to infection and subsequently outbreaks (Poland and Jacobson, 1994; HaralambievaI, Ovsyannikova et al., 2011; Whitaker and Poland, 2014; Haralambieva et al., 2015). Since the mobility of individuals with measles across global, it is hard to avoid imported infections.

The epidemics above can be addressed by vaccinating and treating patients. But now there's no approved vaccine for COVID-19, many people will inevitably die once the emergence of an epidemic occurs. The total number of imported cases of COVID-19 in China reached 2482 as that of August 30th, which poses a threat to the country.

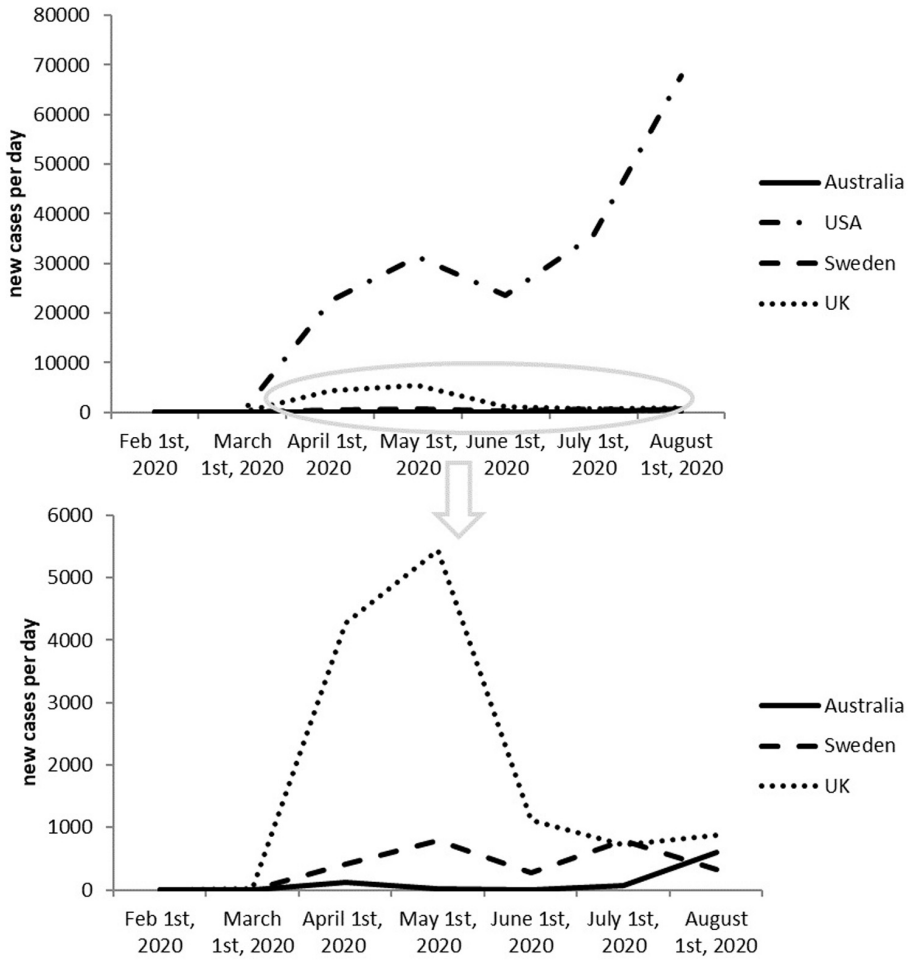


FIGURE 2 | Newly reported cases in last 6 months of United Kingdom, Sweden, United States, and Australia.

Potential Risk of Virus Mutation

To survive and escape the herd immunity, the virus may fight by gene mutation and then the original immune system won't recognize the mutated virus and the herd immunity will thus be ineffective. In this case the viruses can be divided into two categories – DNA and RNA viruses. DNA viruses are stable with low possibility of mutation while RNA viruses are unstable and prone to mutate (Gelderblom, 1996). The SARS-CoV-2 is an RNA virus with a high potential risk of mutation (Phan, 2020). Homologous recombination may result in the cross-species transmission of SARS-CoV-2 (Ji et al., 2020). Population genetic analysis of 103 SARS-CoV-2 genomes showed that SARS-CoV-2 could be categorized into two major types: L and S. The S type was relatively an ancestral version while the L type was found to be more prevalent than S type in the early stage of the outbreak in Wuhan. Besides, 149 sites across 103 sequenced strains were identified (Tang et al., 2020). Recently, a phylogenetic analysis based on 377 complete genomic sequences of the SARS-COV-2 suggested that the virus was actively evolving in human hosts from December 2019 to March 2020 (Li et al., 2020). The highly frequent mutations resulted in at least 5 differentiated SARS-CoV-2 strains and are predicted to enhance the virulence and transmission (Singh et al., 2020; Wang et al., 2020). However, this is a relatively small number of mutations passed through over 300,000 people. At this point, it is still believed that the mutation rate remains low. The analysis from existing study showed that the polyprotein 1ab(pp1ab), the largest protein of coronaviruses, hadn't changed in most isolates during the outbreak (Cárdenas-Conejo et al., 2020). In addition, the critical mechanism for SARS-CoV-2 infection is through S protein binding to the human ACE2 (Lu et al., 2020), and there is no evidence that the binding site of S protein was mutated. Thus the SARS-CoV-2 should be relatively genomically stable.

Epidemiology of COVID-19 in the United Kingdom, Sweden, the United States, and Australia

United Kingdom

The first two cases were confirmed in the United Kingdom on 31 January, 2020. One of them is an international student at

York University and the other is the relative of the student. On March 6th, the number of covid-19 cases in the United Kingdom showed a rapid increase, and the number of new cases in a single day broke new record, reaching 36, with a total of 87 confirmed cases. United Kingdom was the first country that mentioned "herd immunity," but in fact United Kingdom did not adopt "herd immunity" as their strategy against the virus. At present, the total number of confirmed cases in the United Kingdom got stabilized and the outbreak has shown a controllable trend. As of August 30st, 2020, the total number of COVID-19 infections in the United Kingdom reached 334,915. The total number of deaths is 41585.

Sweden

The epidemic in Sweden has come under an intense scrutiny since April, when the country's chief epidemiologist, Dr. Anders Tegnell, announced the country was adopting "herd immunity" to fight against the COVID-19 outbreak. In Sweden, from 31 January to 30 August 2020, there have been 87,072 confirmed cases of COVID-19, along with 5,821 deaths. The number of newly infected cases has become decreased in June but surged again in July. Sweden's death toll is more than three times than that of it's neighbors, Denmark, Finland, and Norway, combined, where lockdown was adopted.

US

Due to the negative response to fight against COVID-19, the epidemic situation in United States had attracted global attention and more and more people began to doubt whether they are adopting "herd immunity" as response strategy or not. Based on the monitoring data of COVID-19 from WHO, we observed that the cases newly infected in the United States has been decreased in June but went up in July, which should be related to the economy reactivation. Currently, the United States has the highest number of confirmed cases in the world, with the huge number of new cases every day.

Australia

Similarly, Australia was also exposed to public scrutiny whether they had adopted "herd immunity". From the WHO data we can see that the cases newly infected in Australia went up again after complete suppression.

It seems like a risky move to adopt herd immunity as a national strategy. And honestly, **Figure 2** shows that herd immunity doesn't seem to be working. In contrast, United Kingdom, has been witnessing newly infected cases decreasing since May as a result of adopting positive measures against COVID-19.

DISCUSSION

Herd immunity can be safely achieved only if it is actively obtained through vaccination. It is not desirable or feasible to achieve herd immunity through natural infection of the population.

To combat with the pandemic of COVID-19, the Chinese government put people's life and health above everything else by taking the most proactive and decisive measures and provided valuable successful experiences for the global "anti-pandemic" battle, such as differentiated isolation strategies and clinical treatments according to different symptoms. In fact, United Kingdom did not adopt "herd immunity" as their strategy against the virus.

We are pious toward our history in order to stay alert and take effective action if needed. COVID-19, SARS and MERS-CoV all belong to human coronaviruses but COVID-19 has caused more deaths than combination of the other two despite the fact that COVID-19 has a lower fatality rate (Mahase, 2020). The history of combating with SARS as well as MERS-CoV has provided much valuable information on the COVID-19 pandemic control. Epidemiological research helps unveil some traits of the viruses but yet there are so many questions remain unanswered.

Figure 3 shows the schematic flow diagram of virus transmission within a population. Susceptible individuals, first become infected and then enter into a latent class, L. They can then progress to a short asymptomatic and potentially infectious stage, I, before the onset of symptoms and the progression to class Y. This diagram assumes that every infected individual eventually goes to hospital and either recovers(HR) or dies(HD) (Riley et al., 2003).

A review on MERS-CoV raised a set of unanswered questions concerning an emerging virus outbreak, such as: the exact routes of the transmission and the incubation period. We don't know yet whether the virus can cause mild and unrecognized diseases and it is also possible that what is reported only represents the tip of an iceberg. The same questions also exist with COVID-19 (Al-Tawfiq, 2013).

However, the strict isolation strategy adopted by China was proved to be effective in the fight against the COVID-19 outbreak. Although the epidemic is currently recurring in some cities at

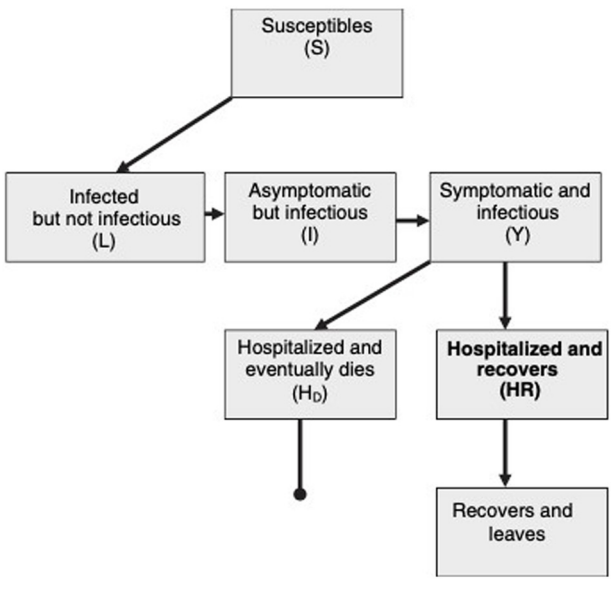


FIGURE 3 | Schematic flow diagram of SARS transmission within a population (Riley et al., 2003).

present, all of them are within controllable range. The result of South Korea's fight against the epidemic also confirmed the effectiveness of the isolation strategy.

In contrast, India has not implemented strict isolation interventions. Since May, the number of new cases per day has continued to increase, even reaching to 70,000. Currently, the total number of cases reached to 3.4 million, ranking third in the world. The same situation also occurs in Brazil and Mexico, which rank second and eighth globally in total cases.

Iran and Iraq had already controlled the epidemic through strict isolation strategy in the early stage. Recently, due to deregulation and imported cases, the epidemic rebounded again and started to get a little out of control.

Taken together, before the emergence of vaccines, isolation is the best and most effective way to fight against the pandemic. The COVID-19 pandemic has been a global public health event, and no country can survive alone. We need to prepare for long-term battles, emphasizing adopting strict isolation measures in severe countries, and resuming work and production without deregulation in countries where the epidemic has improved.

AUTHOR CONTRIBUTIONS

SL, HW, and N-NL contributed to conception and design of the study. YX and LZ wrote the first draft of the manuscript. JT wrote sections of the manuscript. ZZ, JL, YC, AZ, LH, and ZL contributed to the acquisition of data for the work. All authors contributed to manuscript revision, read and approved the submitted version.

FUNDING

This study was supported by grants from the National Key R&D Program of China (2018YFC2000700), the National Natural Science Foundation (81874266, 81673183, 81630086, 81572053, 81971993, and 31900129), the Key Research Program (ZDRW-ZS-2017-1) of the Chinese Academy of Sciences, the Major Science and Technology Innovation Program of Shanghai Municipal Education Commission (2019-01-07-00-01-E00059), the Program for Young Eastern Scholars at Shanghai Institutions of Higher Learning (Program QD2018016); the Shanghai Pujiang Program (18PJ1406600), the Medicine and Engineering Interdisciplinary Research Fund of Shanghai Jiao Tong University (YG2020YQ06 and YG2019QNB39), and the Innovative research team of high-level local universities in Shanghai.

ACKNOWLEDGMENTS

We thank all the lab members in the School of Public Health, Shanghai Jiao Tong University School of Medicine for their help in discussions and with the preparation of the manuscript.

REFERENCES

Adams, D. A., Thomas, K. R., Jajosky, R. A., Foster, L., Baroi, G., Sharp, P., et al. (2017). Summary of notifiable infectious diseases and conditions - United States, 2015. *MMWR* 64, 1–143. doi: 10.15585/mmwr.mm6453a1

Al-Tawfiq, J. A. (2013). Middle east respiratory syndrome-coronavirus infection: an overview. *J. infect. Public Health* 6, 319–322. doi: 10.1016/j.jiph.2013.06.001

Assaad, F. (1983). Measles: summary of worldwide impact. *Rev. Infect. Dis.* 5, 452–459. doi: 10.1093/clinids/5.3.452

Black, F. L. (1982). The role of herd immunity in control of measles. *Yale J. Biol. Med.* 55, 351–360.

Brisson, M., Benard, E., Drolet, M., Bogaards, J. A., Baussano, I., Vanska, S., et al. (2016). Population-level impact, herd immunity, and elimination after human papillomavirus vaccination: a systematic review and meta-analysis of predictions from transmission-dynamic models. *Lancet. Public Health* 1, e8–e17.

Cameron, R. L., Kavanagh, K., Pan, J., Love, J., Cuschieri, K., Robertson, C., et al. (2016). Human papillomavirus prevalence and herd immunity after introduction of vaccination program, Scotland, 2009-2013. *Emerg. Infect. Dis.* 22, 56–64. doi: 10.3201/eid2201.150736

Cárdenas-Conejo, Y., Liñan-Rico, A., García-Rodríguez, D. A., Centeno-Leija, S., and Serrano-Posada, H. (2020). An exclusive 42 amino acid signature in pp1ab protein provides insights into the evolutive history of the 2019 novel human-pathogenic coronavirus (SARS-CoV-2). *Jo. Med. Virol.* 92, 688–692.

CDC (1993). Measles–United States, 1992. *MMWR* 42, 378–381.

CDC (1922-2018). *National Notifiable Diseases Surveillance System. Surveillance and Reporting: Pertussis Cases by Year, passive reports to Public Health Service*. Atlanta, GA: CDC.

de Alwis, R., Williams, K. L., Schmid, M. A., Lai, C. Y., Patel, B., Smith, S. A., et al. (2014). Dengue viruses are enhanced by distinct populations of serotype cross-reactive antibodies in human immune sera. *PLoS pathogens* 10:e1004386. doi: 10.1371/journal.ppat.1004386

Debbink, K., Lindesmith, L. C., Ferris, M. T., Swanstrom, J., Beltramello, M., Corti, D., et al. (2014). Within-host evolution results in antigenically distinct GII.4 noroviruses. *J. Virol.* 88, 7244–7255. doi: 10.1128/jvi.00203-14

Eyler, J. M. (2003). Smallpox in history: the birth, death, and impact of a dread disease. *J. Lab. Clin. Med.* 142, 216–220. doi: 10.1016/s0022-2143(03)00102-1

Ferguson, N. M., Laydon, D., Nedjati-Gilani, G., Imai, N., Ainslie, K., Baguelin, M., et al. (2020). *Impact of Non-Pharmaceutical Interventions (NPIs) to Reduce COVID-19 Mortality and Healthcare Demand*. Available online at: https://www.imperial.ac.uk/media/imperial-college/medicine/sph/ide/gida-fellowships/Imperial-College-COVID19-NPI-modelling-16-03-2020.pdf (Accessed March 16, 2020)

Fine, P., Eames, K., and Heymann, D. L. (2011). "Herd immunity": a rough guide. *Clin. Infect. Dis.* 52, 911–916. doi: 10.1093/cid/cir007

Fine, P. E. (1993). Herd immunity: history, theory, practice. *Epidemiol. Rev.* 15, 265–302. doi: 10.1093/oxfordjournals.epirev.a036121

Fine, P. E. M., Mulholland, K., Scott, J. A., and Edmunds, W. J. (2018). "Community protection," in *Vaccines*, 7th Edn, eds S. A. Plotkin, W. A. Orenstein, P. A. Offit, and K. M. Edwards (Amsterdam: Elsevier), 1512–1531.

Gelderblom, H. R. (1996). "Structure and classification of viruses," in *Medical Microbiology*, ed. S. Baron (Galveston, TX: University of Texas Medical Branch at Galveston).

Haralambieva, I. H. I, Ovsyannikova, G., O'Byrne, M., Pankratz, V. S., Jacobson, R. M., and Poland, G. A. (2011). A large observational study to concurrently assess persistence of measles specific B-cell and T-cell immunity in individuals following two doses of MMR vaccine. *Vaccine* 29, 4485–4491. doi: 10.1016/j.vaccine.2011.04.037

Haralambieva, I. H., Kennedy, R. B. I, Ovsyannikova, G., Schaid, D. J., and Poland, G. A. (2019). Current perspectives in assessing humoral immunity after measles vaccination. *Expert Rev. vaccines* 18, 75–87. doi: 10.1080/14760584.2019.1559063

Haralambieva, I. H., Kennedy, R. B. I, Ovsyannikova, G., Whitaker, J. A., and Poland, G. A. (2015). Variability in humoral immunity to measles vaccine: new

developments. *Trends Mol. Med.* 21, 789–801. doi: 10.1016/j.molmed.2015.10.005

He, H., Chen, E. F., Li, Q., Wang, Z., Yan, R., Fu, J., et al. (2013). Waning immunity to measles in young adults and booster effects of revaccination in secondary school students. *Vaccine* 31, 533–537. doi: 10.1016/j.vaccine.2012.11.014

Ji, W., Wang, W., Zhao, X., Zai, J., and Li, X. (2020). Cross-species transmission of the newly identified coronavirus 2019-nCoV. *J. Med. Virol.* 92, 433–440. doi: 10.1002/jmv.25682

Kang, H. J., Han, Y. W., Kim, S. J., Kim, Y. J., Kim, A. R., Kim, J. A., et al. (2017). An increasing, potentially measles-susceptible population over time after vaccination in Korea. *Vaccine* 35, 4126–4132. doi: 10.1016/j.vaccine.2017.06.058

Katz, S. L., and Hinman, A. R. (2004). Summary and conclusions: measles elimination meeting, 16-17 March 2000. *J. Infect. Dis.* 189(Suppl. 1), S43–S47.

Lane, J. M. (2006). Mass vaccination and surveillance/containment in the eradication of smallpox. *Curr. Top. Microbiol. Immunol.* 304, 17–29. doi: 10.1007/3-540-36583-4_2

Li, Y., Liu, B., Cui, J., Wang, Z., Shen, Y., Xu, Y., et al. (2020). Similarities and evolutionary relationships of COVID-19 and related viruses. *arXiv [Preprint]*. Available online at: https://arxiv.org/abs/2003.05580 (accessed March 26, 2020).

Lu, R., Zhao, X., Li, J., Niu, P., Yang, B., Wu, H., et al. (2020). Genomic characterisation and epidemiology of 2019 novel coronavirus: implications for virus origins and receptor binding. *Lancet* 395, 565–574. doi: 10.1016/s0140-6736(20)30251-8

Mahase, E. (2020). Coronavirus covid-19 has killed more people than SARS and MERS combined, despite lower case fatality rate. *BMJ* 368:m641. doi: 10.1136/bmj.m641

Mallory, M. L., Lindesmith, L. C., and Baric, R. S. (2018). Vaccination-induced herd immunity: successes and challenges. *J. allergy Clin. Immunol.* 142, 64–66. doi: 10.1016/j.jaci.2018.05.007

McNabb, S. J., Jajosky, R. A., Hall-Baker, P. A., Adams, D. A., Sharp, P., Anderson, W. J., et al. (2007). Summary of notifiable diseases — United States, 2005. *MMWR* 54, 1–92.

Midgley, C. M., Bajwa-Joseph, M., Vasanawathana, S., Limpitikul, W., Wills, B., Flanagan, A., et al. (2011). An in-depth analysis of original antigenic sin in dengue virus infection. *J. Virol.* 85, 410–421. doi: 10.1128/jvi.01826-10

Payne, S. (2017). *Viruses: From Understanding to Investigation*. Cambridge: Academic Press.

Peeples, L. (2019). Rethinking herd immunity. *Nature Med.* 25, 1178–1180. doi: 10.1038/s41591-019-0515-2

Phadke, V. K., Bednarczyk, R. A., Salmon, D. A., and Omer, S. B. (2016). Association between vaccine refusal and vaccine-preventable diseases in the united states: a review of measles and pertussis. *JAMA* 315, 1149–1158. doi: 10.1001/jama.2016.1353

Phan, T. (2020). Genetic diversity and evolution of SARS-CoV-2. *Infect. Genet. Evol.* 81:104260. doi: 10.1016/j.meegid.2020.104260

Pica, N., and Palese, P. (2013). Toward a universal influenza virus vaccine: prospects and challenges. *Annu. Rev. Med.* 64, 189–202. doi: 10.1146/annurev-med-120611-145115

Poland, G. A., and Jacobson, R. M. (1994). Failure to reach the goal of measles elimination. Apparent paradox of measles infections in immunized persons. *Arch. Intern. Med.* 154, 1815–1820. doi: 10.1001/archinte.1994.00420160048006

Ribeiro, G. S., Hamer, G. L., Diallo, M., Kitron, U., Ko, A. I., and Weaver, S. C. (2020). Influence of herd immunity in the cyclical nature of arboviruses. *Curr. Opin. Virol.* 40, 1–10. doi: 10.1016/j.coviro.2020.02.004

Riley, S., Fraser, C., Donnelly, C. A., Ghani, A. C., Abu-Raddad, L. J., Hedley, A. J., et al. (2003). Transmission dynamics of the etiological agent of SARS in Hong Kong: impact of public health interventions. *Science* 300, 1961–1966. doi: 10.1126/science.1086478

Singh, H., Singh, J., Khubaib, M., Jamal, S., Sheikh, J. A., Kohli, S., et al. (2020). Mapping the genomic landscape & diversity of COVID-19 based on >3950 clinical isolates of SARS-CoV-2: likely origin & transmission dynamics of isolates sequenced in India. *Indian J. Med. Res.* 151, 474–478.

Smiianov, V. A., Kurhanska, V. A., and Smiianova, O. I. (2019). Measles outbreaks: they are preventable but keep progressing dangerously. *Wiadomosci lekarskie* 72, 2145–2148.

Smith, D. R. (2019). Herd Immunity. The veterinary clinics of North America. *Food Anim. Pract.* 35, 593–604. doi: 10.1016/j.cvfa.2019.07.001

Sugerman, D. E., Barskey, A. E., Delea, M. G. I, Ortega-Sanchez, R., Bi, D., Ralston, K. J., et al. (2010). Measles outbreak in a highly vaccinated population, San Diego, 2008: role of the intentionally undervaccinated. *Pediatrics* 125, 747–755. doi: 10.1542/peds.2009-1653

Tang, X., Wu, C., Li, X., Song, Y., Yao, X., Wu, X., et al. (2020). On the origin and continuing evolution of SARS-CoV-2. *Natl. Sci. Rev.* 3: nwaa036.

Theves, C., Crubezy, E., and Biagini, P. (2016). History of smallpox and its spread in human populations. *Microbiol. Spectr.* 4:1.

Topley, W. W., and Wilson, G. S. (1923). The spread of bacterial infection. the problem of herd-immunity. *J. Hyg.* 21, 243–249. doi: 10.1017/s0022172400031478

Tounkara, K., and Nwankpa, N. (2017). Rinderpest experience. *Rev. Sci. Tech.* 36, 569–578.

Wang, M., Li, M., Ren, R., Li, L., Chen, E. Q., Li, W., et al. (2020). International expansion of a novel SARS-CoV-2 mutant. *J. Virol.* 94, e567–e520.

Whitaker, J. A., and Poland, G. A. (2014). Measles and mumps outbreaks in the United States: think globally, vaccinate locally. *Vaccine* 32, 4703–4704. doi: 10.1016/j.vaccine.2014.06.088

World Health Organization. (2020a). *Report of the WHO-China Joint Mission on Coronavirus Disease 2019 (COVID-19)*. Geneva: World Health Organization.

World Health Organization (2020b). *WHO Coronavirus Disease (COVID-19) Dashboard*. Geneva: World Health Organization.

Wu, N. C., Zost, S. J., Thompson, A. J., Oyen, D., Nycholat, C. M., McBride, R., et al. (2017). A structural explanation for the low effectiveness of the seasonal influenza H3N2 vaccine. *PLoS Pathogens* 13:e1006682. doi: 10.1371/journal.ppat.1006682

Zhang, S., Diao, M., Yu, W., Pei, L., Lin, Z., and Chen, D. (2020). Estimation of the reproductive number of novel coronavirus (COVID-19) and the probable outbreak size on the diamond princess cruise ship: a data-driven analysis. *Int. J. Infect. Dis.* 93, 201–204. doi: 10.1016/j.ijid.2020.02.033

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Medicine

SYSTEMATIC REVIEW
published: 24 September 2020
doi: 10.3389/fmed.2020.552991

The Pipeline of Therapeutics Testing During the Emergency Phase of the COVID-19 Outbreak

Marco Canevelli[1,2], Giulia Remoli[2], Federica Trentin[2], Gabriele Riccardi[3], Leonardo Taricciotti[4], Giovanni Risoleo[5], Antonio Ancidoni[1], Giuseppe Bruno[2], Matteo Cesari[6,7], Nicola Vanacore[1] and Valeria Raparelli[8]*

[1] National Center for Disease Prevention and Health Promotion, Italian National Institute of Health, Rome, Italy, [2] Department of Human Neuroscience, Sapienza University of Rome, Rome, Italy, [3] Department of Sense Organ, Sapienza University of Rome, Rome, Italy, [4] Unit of Neurosurgery, IRCSS Fondazione Cà Granda Ospedale Maggiore Policlinico, University of Milan, Milan, Italy, [5] Department of Radiology, ASTT Grande Ospedale Metropolitano Niguarda, University of Milan, Milan, Italy, [6] Department of Clinical Sciences and Community Health, University of Milan, Milan, Italy, [7] Geriatric Unit, IRCCS Istituti Clinici Scientifici Maugeri, Milan, Italy, [8] Department of Experimental Medicine, Sapienza University of Rome, Rome, Italy

OPEN ACCESS

Edited by:
Mohan Jyoti Dutta, Massey University, New Zealand

Reviewed by:
Xiaojiong Jia, Harvard Medical School, United States
Mohamed M. Abdel-Daim, Suez Canal University, Egypt

***Correspondence:**
Marco Canevelli
marco.canevelli@iss.it

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: *17 April 2020*
Accepted: *01 September 2020*
Published: *24 September 2020*

Citation:
Canevelli M, Remoli G, Trentin F, Riccardi G, Taricciotti L, Risoleo G, Ancidoni A, Bruno G, Cesari M, Vanacore N and Raparelli V (2020) The Pipeline of Therapeutics Testing During the Emergency Phase of the COVID-19 Outbreak. Front. Med. 7:552991. doi: 10.3389/fmed.2020.552991

The coronavirus disease 19 (COVID-19) pandemic poses a serious threat to the sustainability of healthcare systems and is currently having a significant effect on living conditions worldwide. No therapeutic agent has yet proven to be effective for the treatment of COVID-19. The management of this disease currently relies on supportive care and the off-label and compassionate use of antivirals and immunomodulators. Nevertheless, there has been a great worldwide effort to progress research and test the efficacy and safety/tolerability profiles of numerous candidate agents that may positively affect the various clinical syndromes associated with COVID-19. In parallel, vaccination and chemoprophylaxis strategies are being investigated. This article provides a summary of interventional studies targeting COVID-19 during the emergency phase of the outbreak to broadly inform clinicians and researchers on what happened and what they can expect in upcoming months. The clinicaltrials.gov database and the European Union (EU) Clinical Trials Register were investigated on March 31, 2020, to identify all ongoing phase 1–4 research protocols testing pharmacological interventions targeting SARS-CoV-2 infection and/or clinical syndromes associated with COVID-19. Overall, six phase 1, four phase 1-2, 14 phase 2, ten phase 2-3, 19 phase 3, and nine phase 4 studies were identified, and the features of these studies are described in the present review. We also provide an updated overview of the change overtime in the pipeline following this emergency phase and based on the current epidemiology of the COVID-19 pandemic.

Keywords: COVID-19, SARS-CoV-2, clinical trials, antivirals, immunomodulators, research protocols, drug development

INTRODUCTION

The coronavirus disease 19 (COVID-19) pandemic has been caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). It poses a serious threat to the sustainability of healthcare systems, with substantial effects on living conditions worldwide. As of April 3, 2020, more than one million COVID-19 cases and around 53,000 deaths have been calculated in 181 countries worldwide (1). In parallel, nearly half of the global population is currently in lockdown.

To date, no therapeutic compound has been proven to be effective for the treatment of COVID-19. In the initial emergency phase of the outbreak, therapeutic management of affected individuals relied on supportive care (2, 3) and on the off-label and compassionate use of a variety of antiviral (e.g., lopinavir/ritonavir, remdesivir, favipiravir) and/or immunomodulator (e.g., chloroquine, hydroxychloroquine, anti-IL-6 inhibitors, steroids) drugs, the efficacy of which had not then been demonstrated (4, 5). Moreover, their safety and tolerability profiles in patients with COVID-19 remains to be clarified (4, 6).

In this pandemic scenario, a great deal of effort is currently being devoted to the identification of novel therapies and prophylactic strategies, with new research protocols registered internationally every week (if not daily) (7). Moreover, the urgent need to move this field forward in response to this ongoing outbreak needs to be counterbalanced by ensuring that the products under investigation are evaluated through scientifically and ethically appropriate studies (8). There are challenging time-frames connected to the process of developing new therapeutic strategies against COVID-19 or repositioning existing compounds with plausible modifying effects on the disease. The clinical course of patients is not yet fully elucidated (9), and there is incomplete data on the underlying pathophysiological mechanisms (10) and potential therapeutic targets.

In this article, we provide a summary of the interventional studies that have been conducted worldwide to test the efficacy and/or safety/tolerability of pharmacological compounds against COVID-19 in the emergency phase of the pandemic.

METHODS

Data Source

Two databases, the clinicaltrials.gov database and the European Union (EU) Clinical Trials Register, constituted the reference sources for the present study. Clinicaltrials.gov is a web-based resource maintained by the US National Library of Medicine and the National Institute of Health that provides information on publicly and privately supported clinical studies. Registration on this database is mandatory for all clinical investigations of any US Food and Drug Administration (FDA)-regulated drug or medical device. However, it also represents a repository for the vast majority of clinical trial protocols conducted worldwide. EU Clinical Trial Register gathers information on ongoing authorized interventional studies in the EU and the European Economic Area (EEA) that are registered in the EU Drug Regulation Authorities Clinical Trials Database (EudraCT).

Search Strategy

The databases were investigated on March 31, 2020, using the following search terms: "COVID-19" OR "SARS-CoV-2" OR "2019 novel coronavirus" OR "2019-nCoV" OR "severe acute respiratory syndrome coronavirus 2" OR "coronavirus." In clinicaltrials.gov, the advanced search function was used to restrict the search to: (i) interventional studies (STUDY TYPE); (ii) "recruiting," "enrolling by invitation," and "active not recruiting" protocols (STATUS: RECRUITMENT); and (iii) phase 1, phase 2, phase 3, phase 4 studies (PHASE).

Two reviewers (L.T. and G.R.) screened the identified protocols to remove duplicates and verify the fulfillment of the following predefined inclusion criteria: (1) targeting SARS-CoV-2 infection and/or clinical syndromes associated with COVID-19; and (2) testing the efficacy and/or safety/tolerability of pharmacological interventions. Studies investigating novel medical devices or diagnostic tools were not considered in the present analysis. Disagreements in the selection were solved by consensus, involving two additional reviewers (M.C. and V.R.). The flow chart in **Figure 1** illustrates the process of protocols' selection.

Data Extraction

The following data were abstracted by three authors (F.T., Ga.R., and Gi.R.) from the selected protocols: NCT (the unique identification code assigned by clinicaltrials.gov) and/or EudraCT codes; study phase; allocation and masking procedures; tested compound(s); way of administration; mechanism of action; primary outcome measure(s); expected primary completion date; expected number of participants; age range of participants; targeted COVID-19 related condition; sponsor; and location.

Clinical syndromes associated with COVID-19 were coded according to the classifications provided by the World Health Organization (WHO) (i.e., mild illness, pneumonia, severe pneumonia, acute respiratory distress syndrome [ARDS], sepsis, and septic shock) (2). When the WHO classification could not be applied or was not specified, the targeted conditions were classified according to the definitions provided in the protocol.

RESULTS

Search Results

A total of 74 protocols were identified through a structured search of the two adopted databases ($n = 57$ on clinicaltrials.gov and $n = 17$ on EU Clinical Trials Register). After the removal of six duplicates, six additional protocols were excluded because not targeting COVID-19. Specifically, five of them were focused on the Middle East Respiratory Syndrome and one on other bacterial or viral infections. Thus, 62 protocols were ultimately retained. The reviewers reported a >90% agreement in the selection process.

Characteristics of the Selected Protocols

Clinical trials involving new drugs are commonly classified into four phases, with some individual trials encompassing more than one phase (e.g., combined phase 1–2). Overall, six phase 1, four phase 1–2, 14 phase 2, ten phase 2–3, 19 phase 3, and nine

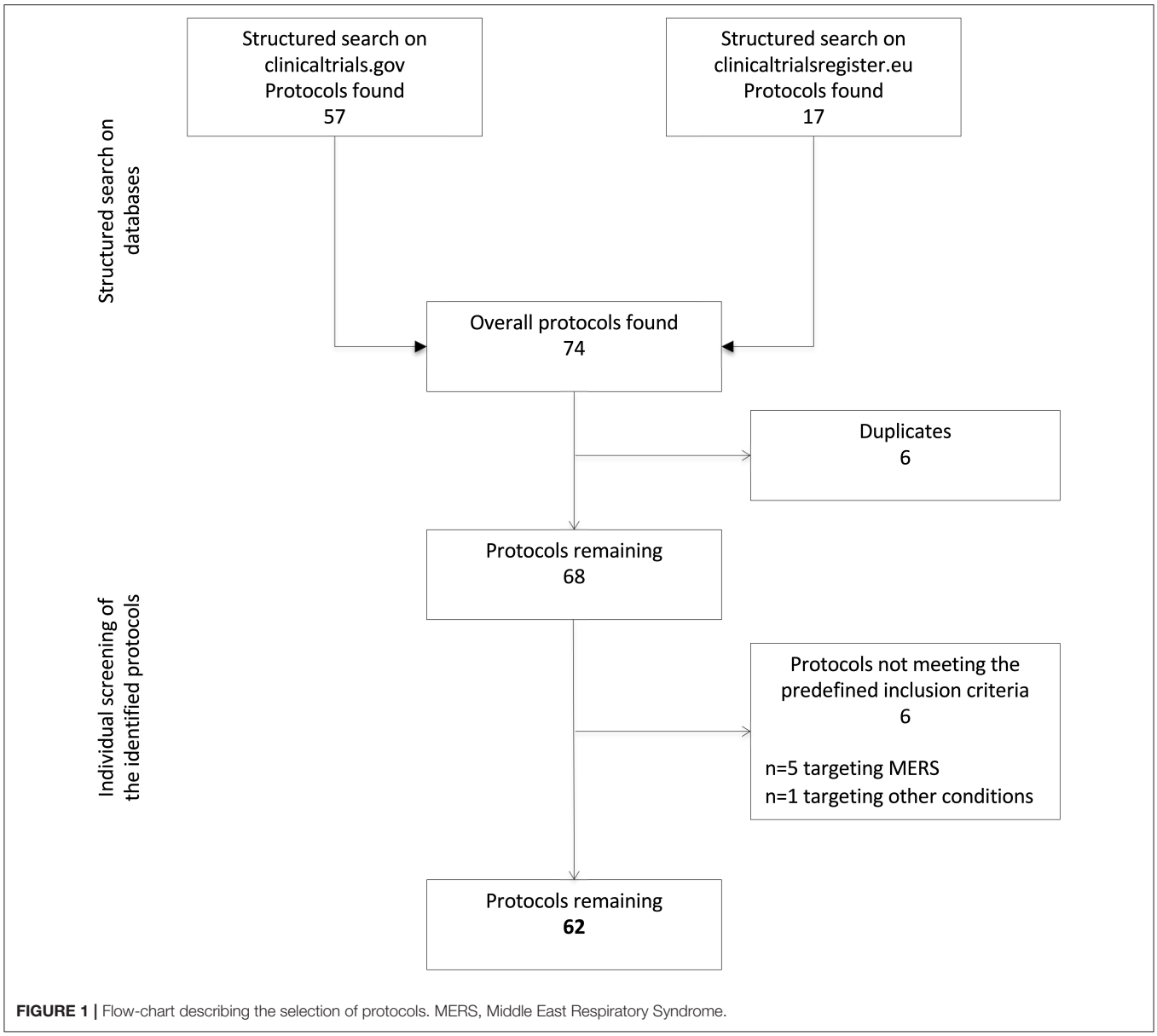


FIGURE 1 | Flow-chart describing the selection of protocols. MERS, Middle East Respiratory Syndrome.

phase 4 studies were identified. Their detailed characteristics are presented in **Tables 1**–**3**.

Most trials were conducted in China ($n = 30$), followed by the US ($n = 10$), Italy ($n = 8$), Germany ($n = 6$), France ($n = 6$), Spain ($n = 5$), and Korea ($n = 5$) (**Figure 2**). Seven trials involved international networks of clinical sites, whereas 55 were run in single countries; 35% of studies ($n = 22$) are multicentric. The majority of studies were funded by non-commercial research institutions (e.g., universities, hospitals, foundations, institutes) while only 11 were sponsored by the biopharma industry. Protocols had a varying duration and are expected to be completed (in terms of primary completion) between April 2020 and July 2023.

Forty-five trials had a randomized design, mostly relying on a parallel assignment of participants. Masking procedures were instead adopted by less than half of the trials ($n = 26$), with nine studies reporting quadruple masking involving both the participants, investigators, care providers, and outcomes assessors. Placebo or standard care were used as comparators in 27 studies, and 14 use one or more active comparators, whereas nine compared different regimens (i.e., dosages and/or duration) of the same treatment.

Tested Interventions

Most protocols ($n = 32$) investigated the efficacy and/or safety profiles of compounds that are expected to act as immune system modulators in COVID-19 associated conditions

TABLE 1 | Characteristics of the selected phase 1, phase 1-2, phase 2, and phase 2-3 protocols targeting COVID-19-related conditions.

Identification Trial Number	Treatment(s) Comparator(s)(if any)	Primary completion	Allocation Assignment	Masking	Primary outcome(s)	Subjects	Age	Condition
Phase 1								
NCT04252118	1. Mesenchymal stem cells IV 2. Conventional treatment	December 20	Non-randomized Parallel	None	- Size of lesion area by chest radiograph or CT (day 0,3,6,10,14,21,28) - Side effects (day 0,3,6,10,14,21,28,90,180)	20	18-70y	Pneumonia*
NCT04313322	1. Wharton's Jelly mesenchymal stem cells IV	June 20	Single group	None	- Improvement of clinical symptoms (week 3) - Side effects measured by chest radiograph (week 3) - RT-PCR results (week 3)	5	≥18y	Infection*
NCT04299724	1. Artificial antigen presenting cells (aAPC) vaccine SC	July 23	Single group	None	- Vaccine events and severe events (days 0-28) - Proportion of subjects with positive T cell response (days 0-28)	100	6m-80y	Healthy subjects Infection*
NCT04313127	1. Ad5-nCoV vaccine (low) IM 2. Ad5-nCoV vaccine (middle) IM 3. Ad5-nCoV vaccine (high) IM	December 20	Non-randomized Sequential	None	- Safety indexes of adverse reactions (days 0-7)	108	18-60y	Healthy subjects
NCT04283461	1. mRNA-1273 vaccine (low) IM 2. mRNA-1273 vaccine (middle) IM 3. mRNA-1273 vaccine (high) IM	June 21	Non-randomized Sequential	None	- Frequency of adverse events and new-onset chronic medical conditions (days 0-394)	45	18-55y	Healthy subjects
NCT04280224	1. Natural killer cells 2. Conventional treatment	September 20	Randomized Parallel	None	- Improvement of clinical symptoms (days 0-28) - Adverse events (days 0-28)	30	18-65y	Pneumonia*
Phase 1-2								
NCT04288102	1. Mesenchymal stem cells IV 2. Placebo	December 20	Randomized Parallel	Yes (PCIOa)	- Size of lesion area and severity of pulmonary fibrosis by chest CT (day 0,6,10,14,28,90)	90	18-75y	Severe pneumonia ARDS
NCT04324996	1. Natural killer (NK) cells IV 2. IL15-NK cells IV 3. NKG2D CAR-NK cells IV 4. ACE2 CAR-NK cells IV 5. NKG2D-ACE2 CAR-NK cells IV	May 20	Randomized Parallel	Yes (PCIOa)	- Clinical response (day 28) - Side effects (day 28)	90	≥18y	Severe pneumonia ARDS Sepsis/septic shock
NCT04276896	1. Synthetic minigene vaccine (LV-SMENP-DC) IV SC	July 23	Single group	None	- Clinical improvement (day 28) - Lower Murray lung injury score (day 7)	100	6m-80y	Healthy subjects Infection*
NCT04275245	1. Meplazumab IV	December 20	Single group	None	- Virological clearance rate using RT-PCR (day 3,7,14)	20	18-75y	Pneumonia*
Phase 2								
NCT04307693	1. Lopinavir/Ritonavir O 2. Hydroxychloroquine O 3. No intervention	May 20	Randomized Parallel	None	- Viral load (day 3,5,7,10,14,18)	150	16-99y	Pneumonia
NCT04280588	1. Fingolimod O 2. No intervention	July 20	Non-randomized Parallel	None	- Change of pneumonia severity on X-ray images (day 5)	30	18-85y	Pneumonia Severe pneumonia
NCT04317092 EudraCT-2020-001110-38	1. Tocilizumab IV	December 20	Single group	None	- Mortality rate (month 1)	330	All	Severe pneumonia ARDS
NCT04279197	1. Fuzheng Huayu O 2. Placebo	December 22	Randomized Parallel	Yes (PI)	- Evaluation of pulmonary fibrosis (CT)(week 24) - Evaluation of lung function improvement (week 24)	136	18-65y	Pulmonary fibrosis*
NCT04305457	1. Nitric oxide IN 2. No intervention	April 21	Randomized Parallel	None	- Reduction in the incidence of patients requiring intubation and mechanical ventilation (day 28)	240	≥18y	Pneumonia Severe pneumonia
NCT04306393	1. Nitric oxide IN 2. No intervention	March 21	Randomized Parallel	Yes (P)	- Change of arterial oxygenation (48 hours)	200	18-99y	ARDS
NCT04269525	1. Umbilical cord derived mesenchymal stem cells IV	April 20	Single group	None	- Oxygenation index (day 14)	10	18-75y	Severe pneumonia ARDS
NCT04264533	1. Vitamin C IV 2. Placebo	September 20	Randomized Parallel	Yes (PCOa)	- Ventilation-free days (day 28)	140	≥18y	Severe pneumonia ARDS

(Continued)

TABLE 1 | Continued

Identification Trial Number	Treatment(s) Comparator(s)(if any)	Primary completion	Allocation Assignment	Masking	Primary outcome(s)	Subjects	Age	Condition
NCT04323527	1. Chloroquine (low) O 2. Chloroquine (high) O	August 20	Randomized Parallel	Yes (PCIOa)	- Absolute mortality (day 28)	440	18-100y	SARS with or without infection*
NCT04276688	1. Lopinavir/Ritonavir O + Ribavirin O + Interferon ß-1b SC 2. Lopinavir/Ritonavir O	January 22	Randomized Parallel	None	- Time to negative nasopharyngeal swab (month 1)	70	≥18y	Infection*
EudraCT-2020-001200-42	1. Camostat mesylate O 2. Placebo	na	Randomized Parallel	Yes (DB)	- Time to clinical improvement (from day 0 to discharge/death)	180	≥18y	Infection*
EudraCT-2020-001023-14	1. Interferon β-1a IN 2. Placebo	na	Randomized	Yes (DB)	- Clinical improvement (day 14)	400	≥18y	Infection*
EudraCT-2020-001224-33	1. Hydroxychloroquine O 2. Placebo	na	Randomized Parallel	Yes (DB)	- Viral clearance (RT-PCR)	220	≥18y	Severe pneumonia
EudraCT-2020-001243-15	1. Itraconazole O 2. Best clinical practice	na	Randomized	None	- Clinical improvement (day 15)	200	≥18y	Pneumonia Severe pneumonia ARDS
Phase 2-3								
NCT04315298	1. Sarilumab (low) IV 2. Sarilumab (high) IV 3. Placebo	March 21	Randomized Parallel	Yes (PCIOa)	- Time to resolution of fever (day 29) - Clinical improvement (day 15)	400	≥18y	Severe pneumonia ARDS Sepsis
NCT04278963	1. Yinhu Qingwen Decoction (low) O 2. Yinhu Qingwen Decoction (high) O	January 21	Randomized Parallel	Yes (PO)	- Mean clinical recovery time (day 28)	300	≥18y	Pneumonia
NCT04275414	1. Bevacizumab IV	April 20	Single group	None	- PaO2 to FiO2 ratio (day 1,3,7)	20	18-80y	ARDS
NCT04322344	1. Escin O 2. Escin IV 3. Standard therapy	June 20	Non-randomized Parallel	Yes (PC)	- Mortality rate (day 30) - Clinical status (day 30)	120	18-75y	Infection*
NCT04323592	1. Methylprednisolone IV	May 20	Single group	None	- Death or ICU admission or Invasive ventilation (composite)(day 28) - Death (day 28) - Admission to ICU (day 28) - Endotracheal intubation (day 28)	104	18-80y	ARDS
NCT04244591	1. Methylprednisolone IV 2. Standard of care	April 20	Randomized Parallel	None	- Lower Murray lung injury score (day 7,14)	80	≥18y	ARDS
NCT04319900	1. Favipiravir O + Chloroquine O 2. Favipiravir O 3. Placebo	April 20	Randomized Parallel	Yes (PC)	- Time of improvement or recovery of respiratory symptoms (day 10) - Number of days virus nucleic acid shedding (day 10) - Frequency of improvement or recovery of respiratory symptoms (day 10)	150	18-75y	Pneumonia Severe pneumonia
EudraCT-2020-001246-18	1. Sarilumab IV 2. Tocilizumab IV 3. Anakinra IV 4. Standard of care	na	Randomized Parallel	None	- Survival without needs of ventilator utilization (day 14) - Cumulative incidence of successful tracheal extubation (day 14) - Clinical improvement (day 4)	1,000	≥18y	Pneumonia Severe pneumonia ARDS
EudraCT-2020-001113-21	1. Lopinavir/Ritonavir O 2. Interferon β-1a IN 3. Dexamethasone IV 4. Hydroxychloroquine O	na	Randomized Parallel	None	- In-hospital mortality (day 28)	2,000	≥18y	Severe pneumonia ARDS
EudraCT-2020-001162-12	1. Sarilumab IV 2. Placebo	na	Randomized Parallel	Yes (DB)	- Time to resolution of fever (day 29) - Clinical improvement (day 15)	460	≥18y	Severe pneumonia ARDS

*IM, intramuscular; IN, inhaled; IV, intravenous; O, oral; SC, subcutaneous; DB, double blind; P, participant; I, investigator; C, care provider; Oa, outcomes assessor. *Not based on the WHO classification of COVID-19 associated conditions.*

TABLE 2 | Characteristics of the selected phase 3 protocols targeting COVID-19-related conditions.

Identification trial number	Treatment(s) Comparator(s)(if any)	Primary completion	Allocation Assignment	Masking	Primary outcome(s)	Subjects	Age	Condition
NCT04292899 EudraCT-2020-000841-15	1. Remdesivir IV (5 days) 2. Remdesivir IV (10 days)	May 20	Randomized Parallel	None	- Proportion with normalization of fever and oxygen saturation (day 14)	2,400	≥18y ≥12y	Pneumonia
NCT04292730 EudraCT-2020-000842-32	1. Remdesivir IV (5 d) 2. Remdesivir IV (10 d) 3. Standard of care	May 20	Randomized Parallel	None	- Proportion of participants discharged by (day 14)	600	≥18y	Pneumonia
NCT04304313	1. Sildenafil O	Mar 20	Single group	None	- Rate of disease remission (day 14) - Rate of entering the critical stage (day 14) - Time of entering the critical stage (day 14)	10	≥18y	Pneumonia Severe pneumonia
NCT04304053	1. Darunavir/Cobicistat O 2. Hydroxychloroquine O 3. Isolation	June 20	Cluster-RCT Randomized Parallel	None	- Incidence of secondary COVID-19 cases among contacts (day 14) (chemoprophylaxis)	3,040	≥18y	Healthy subjects Infection*
NCT04252664	1. Remdesivir O 2. Placebo	April 20	Randomized Parallel	Yes (PCIOa)	- Time to clinical recovery (day 28)	308	≥18y	Pneumonia
NCT04320238	1. Recombinant human Interferon α-1b IN 2. Recombinant human Interferon α-1b IN + Thymosin α1 SC	May 20	Non-randomized Parallel	None	- New-onset COVID-2019 (week 6)	2,944	18–65y	Healthy health care providers
NCT04261270	1. ASC09F + Oseltamivir O 2. Ritonavir + Oseltamivir O 3. Oseltamivir O	May 20	Randomized Parallel	Yes (P)	- Rate of comprehensive adverse outcome (day 14)	60	18–55y	Pneumonia
NCT04322682	1. Colchicine O 2. Placebo	September 20	Randomized Parallel	Yes (P)	- Composite of death or the need for hospitalization due to COVID-19 infection (day 30)	6,000	≥40y	Infection*
NCT04315948 EudraCT-2020-000936-23	1. Remdesivir IV 2. Lopinavir/Ritonavir O 3. Lopinavir/Ritonavir O + Interferon β-1a SC 4. Hydroxychloroquine O 5. Standard of care	March 23	Randomized Parallel	None	- Clinical improvement (day 15)	3,100	≥18y	Pneumonia Severe pneumonia ARDS
NCT04280705 EudraCT-2020-001052-18	1. Remdesivir IV 2. Placebo	April 23	Randomized Parallel	Yes (PI)	- Clinical improvement (day 15)	440	18–99y	Pneumonia Severe pneumonia ARDS
NCT04257656	1. Remdesivir IV 2. Placebo	April 20	Randomized Parallel	Yes (PCIO)	- Time until clinical improvement (day 28)	453	≥18y	Severe pneumonia ARDS
NCT04252274	1. Darunavir/Cobicistat O 2. Conventional treatment	August 20	Randomized Parallel	None	- Virological clearance (day 7)	30	All	Pneumonia Severe pneumonia
NCT04320277	1. Baricitinib + Ritonavir O 2. Ritonavir O and/or Hydroxychloroquine O	April 20	Non-randomized Crossover	None	- Percentage of ICU admission in patients vs. controls (week 2)	60	18-85y	Pneumonia
NCT04308668	1. Hydroxychloroquine O 2. Placebo	April 20	Randomized Parallel	Yes (PCIOa)	- Incidence of COVID-19 in asymptomatic subjects (day 14) - Change in COVID-19 Severity (day 14) among symptomatic:	3,000	≥18y	Healthy subjects Infection*

(Continued)

TABLE 2 | Continued

Identification trial number	Treatment(s) Comparator(s)(if any)	Primary completion	Allocation Assignment	Masking	Primary outcome(s)	Subjects	Age	Condition
NCT04282902	1. Pirfenidone O 2. Standard of care	April 20	Randomized Parallel	None	- Laboratory, imaging and clinical improvement (week 4)	294	≥18y	Severe pneumonia
NCT03680274	1. Vitamin C IV 2. Placebo	December 21	Randomized Parallel	Yes (PCIOa)	- Deceased participants or with persistent organ dysfunction (day 28)	800	≥18y	Sepsis
NCT03808922	1. DAS181 IN 2. Placebo	April 21	Randomized Parallel	Yes (PCIO)	- Clinical status improvement (day 14) (sub-study)	250 (main study)	All	Severe pneumonia
EudraCT-2020-000982-18	1. Hydroxychloroquine O 2. Standard of care	na	Randomized	None	- In-hospital mortality	443	≥18y	Severe pneumonia ARDS Sepsis/septic shock
EudraCT-2020-000890-25	1. Hydroxychloroquine O	na	Single group	None	- Results of SARS-CoV2 virus detection (day 1,4,7,14)	25	≥12y	Infection*

*IN, inhaled; IV, intravenous; O, oral; SC, subcutaneous; P, participant; I, investigator; C, care provider; Oa, outcomes assessor. *Not based on the WHO classification of COVID-19 associated conditions.*

(**Figure 3**). These compounds included vaccines ($n = 5$), cell-based therapies ($n = 6$; e.g., mesenchymal stem cells, natural killer cells), antimalarial drugs ($n = 9$; e.g., chloroquine and hydroxychloroquine), corticosteroids ($n = 4$), interleukin inhibitors, and interferons. Twenty-two studies have been testing antiviral agents such as antiretroviral protease inhibitors (e.g., darunavir, lopinavir, ritonavir), neuraminidase inhibitors (e.g., oseltamivir), nucleotide analogs (e.g., remdesivir), and broad-spectrum antivirals. The remaining trials were designed to investigate other potential adjuvant therapies such as nitric oxide, antioxidants, phosphodiesterase inhibitors. Finally, seven studies have been evaluating the combinations of substances with both immunomodulant and antiviral properties.

Most of the selected primary outcome measures referred to clinical endpoints (e.g., mortality rates, clinical improvement/remission, hospital discharge, intensive care unit admission, ventilation-free days). A sizeable proportion of studies ($n = 24$) incorporated laboratory (e.g., viral clearance/load) or radiological (e.g., change of pneumonia severity on X-ray or CT) changes as primary endpoints. A residual number of trials ($n = 7$) were instead primarily aimed at exploring the safety and tolerability profiles of the tested interventions.

Targeted Conditions

A total of 41,110 participants will tentatively be recruited in the selected protocols, with sample sizes widely ranging between five and 6,800 subjects.

The entire clinical spectrum of COVID-19, ranging from infection with mild symptoms to sepsis complicated by shock, was targeted by the studies in the emergency phase of the COVID-19 outbreak. It also planned to recruit healthy subjects or individuals exposed to higher risk (e.g., healthcare providers or household contact).

Early phase studies (**Table 1**) preliminarily tested the tolerated dose, the safety, and efficacy of candidate agents in small representative groups. The target population was composed by healthy subjects ($n = 4$), individuals with laboratory confirmed infection without a clear WHO definition of the clinical syndrome ($n = 6$), patients with pneumonia ranging from mild to severe ($n = 19$), and more severe/critical clinical syndromes including acute respiratory distress syndrome (ARDS) and sepsis ($n = 14$). In the late phases studies (**Tables 2**, **3**) that are testing on a large scale those agents with documented safety and evidence of preliminary efficacy in the earlier phases, the target participants were largely represented by healthy or at-risk subjects with infection ($n = 7$), patients with mild/severe pneumonia ($n = 20$), and patients with more severe/critical clinical syndrome ($n = 10$).

DISCUSSION

Although the first COVID-19 cases were reported just 4 months ago (11), there has been an unprecedented response from the international community. The findings of several interventional studies have already been disseminated (5, 12). Encouragingly, a relevant number of clinical trials have explored safe and effective therapeutics to face the pandemic, enrolling individuals with

TABLE 3 | Characteristics of the selected phase 4 protocols targeting COVID-19-related conditions.

Identification trial number	Treatment(s) Comparator(s)(if any)	Primary completion	Allocation Assignment	Masking	Primary outcome(s)	Subjects	Age Years	Condition
NCT04308317	1. Tetrandrine O 2. Standard of care	March 21	Randomized Parallel	None	- Death event (week 12)	60	18–75y	Pneumonia Severe pneumonia
NCT04326920 EudraCT-2020-001254-22	1. Sargramostim IN or IV 2. Placebo	October 20	Randomized Parallel	None	- Improvement in oxygenation (day 5)	80	18–80y	Severe pneumonia ARDS
NCT04255017	1. Abidol hydrochloride O 2. Oseltamivir O 3. Lopinavir/Ritonavir O 4. Symptomatic treatment	June 20	Randomized Parallel	Yes (P)	- Rate of clinical remission (week 2) - Time of lung imaging recovery (week 2)	400	≥18y	Pneumonia Severe pneumonia ARDS
NCT04254874	1. Abidol hydrochloride O 2. Abidol hydrochloride O + Interferon (PegIFNα-2b) IV	June 20	Randomized Parallel	Yes (P)	- Rate of clinical remission (week 2) - Time of lung imaging recovery (week 2)	100	≥18y	Pneumonia Severe pneumonia ARDS
NCT04263402	1. Methylprednisolone (<40mg) IV 2. Methylprednisolone(40-80mg) IV	June 20	Randomized Parallel	Yes (P)	- Rate of disease remission (day 7) - Rate and time of entering the critical stage (respiratory failure or multiorgan failure)(day 7)	100	≥18y	Severe pneumonia ARDS
NCT02735707	1. No antiviral 2. Lopinavir/Ritonavir O 3. Hydroxychloroquine O 4. Hydroxychloroquine O + Lopinavir/Ritonavir O 5. No immune modulators 6. Interferon β-1a IV 7. Anakinra IV	December 21	Randomized Factorial	None	- All cause death (day 90) - Days alive and outside of ICU (day 21)	6,800 (main study)	≥18y	Sever pneumonia ARDS Sepsis/septic shock
NCT04252885	1. Lopinavir/Ritonavir O 2. Arbidol O 3. Standard of care	May 20	Randomized Parallel	None	- The rate of virus inhibition (in nose/throat swab) (day 0,2,4,7,10,14,21)	125	18–80y	Infection*
2020-001010-38	1. Hydroxychloroquine O 2. Standard of care		Randomized	None	- Rate of decline in SARS-CoV-2 viral load in nasopharyngeal samples (96 h)	200	≥18y	Pneumonia
EudraCT-2020-000919-69	1. Bacillus Calmette-Guérin vaccination ID		Randomized	Yes (DB)	- Number of days of unplanned absenteeism for any reason (hospital personnel) (days 0–180)	1,000	≥18y	Healthcare providers

*ID, intradermal; IN, inhaled; IV, intravenous; O, oral; DB, double blind; P, participant. *Not based on the WHO classification of COVID-19 associated conditions.*

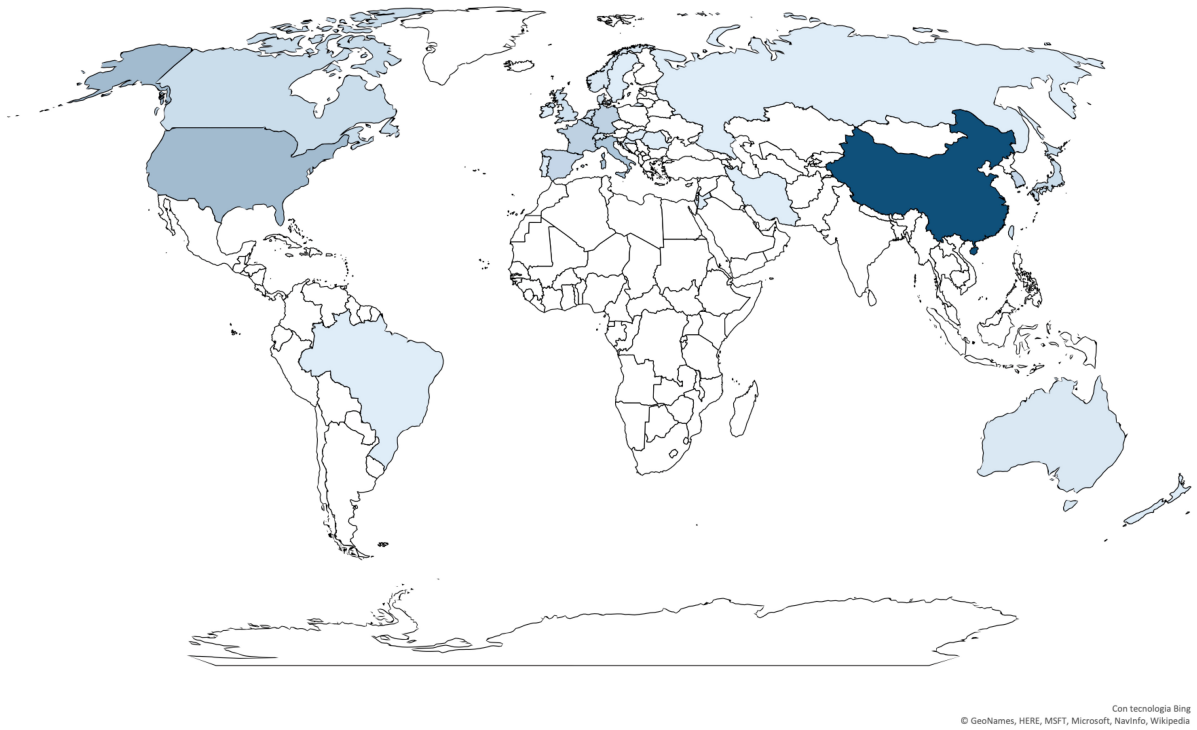


FIGURE 2 | Geographic location of the selected protocols.

COVID-19 worldwide, and some of these trials will publish the results as early as in the next few weeks/months. This emerging evidence will largely be concerned with hard outcomes such as mortality (adopted as the primary endpoint in ten studies), access to intensive care units, clinical remission, and will therefore have profound clinical implications.

This review provides an overview of studies in the emergency phase of the outbreak, utilizing the two most common open access protocol registries in the US and Europe, with the aim of informing clinicians and researchers on what they can expect in the upcoming months. Of note, is the fact that we restricted our focus to only two clinical trial registries and we are aware that this might potentially underestimate the current situation. When a broader search is conducted by including most of the existing national and international databases, the number of ongoing studies is much higher and needs to be constantly updated (13). Accordingly, coalitions/networks have recently been launched to provide frequently updated resources (e.g., living systematic reviews) summarizing the characteristics of research protocols targeting COVID-19 (7, 13). These initiatives are particularly welcomed, as they potentially allow for the coordination of a multinational research effort and better allocation of the available research resources.

As expected, interventional studies were largely performed and promoted in those countries where the outbreak has already significantly affected the community and the healthcare system. The inclusion criteria of the studies was designed to target the entire spectrum of clinical syndromes associated with COVID-19 at the time the study was conducted, namely asymptomatic status, mild illness, pneumonia, ARDS, and septic complications. The opportunity to include the clinical struggles for different categories of patients was also implemented. Several trials were instead focused on the vaccination and chemoprophylaxis of healthy individuals. Two studies were specifically dedicated to health care providers, consistently with their established vulnerability in the COVID-19 pandemic (14). These studies are very much needed, as in some countries the number of healthcare providers with infection is rapidly increasing due to a shortage of personal protective equipment, in parallel with the high demand for care that usually occurs during a pandemic (15). Currently, slowing the spread of the SARS-CoV-2 relies on measures of social distancing and recommended changes to lifestyle and behavior that have unmeasurable consequences on the life of individuals and communities, not to mention the economic crisis that countries face. In light of this, it is pivotal to cooperate and optimize the effort for a common solution starting from the systematic recruitment of patients to complete the ongoing trials.

Based on the registered information, some protocols will probably provide *proof-of-concept* evidence supporting the design of large-scale clinical trials. Conversely, some of the ongoing phase 3 randomized controlled trials and phase 4 post marketing studies seem already adequately informed to be able to

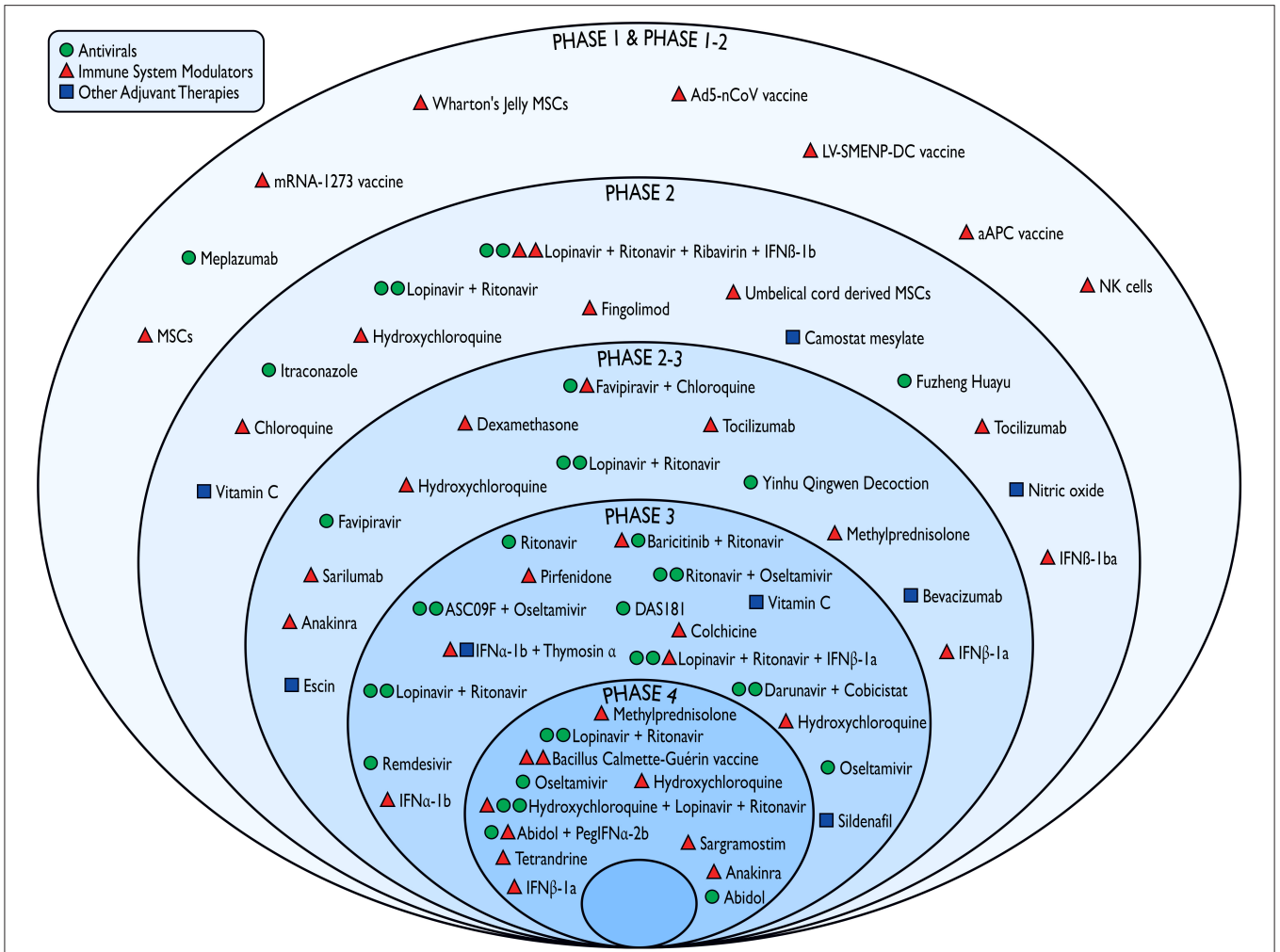


FIGURE 3 | Pharmacological interventions against COVID-19 tested in the emergency phase of the outbreak according to the study phase and mechanism of action. IFN, interferon; MSCs, mesenchymal stem cells; NK, natural killers.

draw either positive or negative conclusions on the efficacy and safety/tolerability of pharmacological compounds with different mechanisms of action. Of note, is the fact that some trials are adopting adaptive designs, allowing them to rapidly accept or reject multiple experimental therapies, which is especially promising in the current outbreak scenario (4).

The major limitation of our study is related to the extremely dynamic evolution of knowledge on the topic. As mentioned, an incredible number of trials have been proposed on COVID during the past weeks and it is likely that this number will rapidly and exponentially increase in the next months, especially given the more consistent dissemination of the coronavirus in different regions of the world. In this regard, since April 1, 2020, 585 new protocols have been registered on the clinicaltrials.gov database (search updated to August 18, 2020) with an expected overall number of around 375,000 participants. As compared with the emergency phase, a greater proportion of phase 1 and 2 studies are currently active (70.2 vs. 54.8%). An increase in the percentage of industry-funded trials (34.2 vs. 17.7%) and of studies adopting a randomized design (85.3 vs. 72.6%) has been observed. As expected by the changes that have occurred in the epidemiology of COVID-19, the US and Europe persist as the main recruiting sites while centers in South America, India, and Africa have recently started to contribute. It is noteworthy that, due to a better understanding of the pathophysiological mechanisms of the disease (10), there are a relevant number of novel compounds, mostly acting as immunomodulators, that are being tested (e.g., ruxolitinib, colchicine, heparins, mavirilumab, ivermectin). These were not present nor in the pipeline at the end of March 2020. Moreover, 47 protocols are currently investigating the efficacy and safety profiles of vaccines whereas 67 focused on convalescent plasma therapies. As of August 18, 2020, the (negative) findings of four of the studies that started in the emergency phase have already been published (i.e., three testing remdesivir [NCT04292899, NCT04292730, NCT04257656] and one testing hydroxychloroquine [NCT04308668]) (16–19).

In conclusion, the present analysis provides an account for researchers and clinicians for them to understand present

research and envision the future of therapeutics testing for the management of the COVID-19 pandemic.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article.

AUTHOR CONTRIBUTIONS

MCa and VR: study design, data analysis, and writing of the manuscript. GRe: study design, data collection, and drafting of the manuscript. FT, GRic, LT, GRis, and AA: data collection. GB, MCe, and NV: data interpretation and drafting of the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

MCa is supported by a research grant of the Italian Ministry of Health for the project Dementia in immigrants and ethnic minorities living in Italy: clinical-epidemiological aspects and public health perspectives (ImmiDem) (GR-2016-02364975). VR was supported by the Scientific Independence of Young Researchers Program (RBSI14HNVT), Italian Ministry of Education, University and Research (MIUR), Rome, Italy.

ACKNOWLEDGMENTS

We thank Marco Moroni for the realization of **Figure 3**. We thank Dr. Giuseppe Traversa (National Centre for Drug Research and Evaluation, Italian National Institute of Health) for revising the manuscript for intellectual content.

REFERENCES

1. Johns Hopkins Coronavirus Resource Center. *COVID-19 Map*. Available online at: https://coronavirus.jhu.edu/map.html (accessed April 3, 2020)
2. World Health Organization. Clinical management of severe acute respiratory infection when COVID-19 is suspected. *Pediatria Medycyna Rodzinna.* (2020) 16:9–26. doi: 10.15557/PiMR.2020.0003
3. Poston JT, Patel BK, Davis AM. Management of critically Ill adults with COVID-19. *JAMA*. (2020). doi: 10.1001/jama.2020.4914. [Epub ahead of print].
4. Kalil AC. Treating COVID-19—Off-label drug use, compassionate use, and randomized clinical trials during pandemics. *JAMA*. (2020). doi: 10.1001/jama.2020.4742. [Epub ahead of print].
5. Cao B, Wang Y, Wen D, Liu W, Wang J, Fan G, et al. A Trial of Lopinavir-Ritonavir in Adults Hospitalized with Severe Covid-19. *N Engl J Med.* (2020) 382:1787–1799. doi: 10.1056/NEJMc2008043
6. Russell CD, Millar JE, Baillie JK. Clinical evidence does not support corticosteroid treatment for 2019-nCoV lung injury. *Lancet.* (2020) 395:473–5. doi: 10.1016/S0140-6736(20)30317-2
7. Maguire BJ, Guérin PJ. A living systematic review protocol for COVID-19 clinical trial registrations. *Wellcome Open Res.* (2020) 5:60. doi: 10.12688/wellcomeopenres.15821.1
8. European Medicines Agency. *Points to Consider on Implications of Coronavirus Disease (COVID-19) on Methodological Aspects of Ongoing Clinical Trials.* Amsterdam: European Medicines Agency (2020).
9. Zhou F, Yu T, Du R, Fan G, Liu Y, Liu Z, et al. Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: a retrospective cohort study. *Lancet.* (2020) 395:1054–62. doi: 10.1016/S0140-6736(20)30566-3
10. Kabir MT, Uddin MS, Hossain MF, Abdulhakim JA, Alam MA, Ashraf GM, et al. nCOVID-19 pandemic: from molecular pathogenesis to potential investigational therapeutics. *Front Cell Dev Biol.* (2020) 8:616. doi: 10.3389/fcell.2020.00616
11. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet.* (2020) 395:497–506. doi: 10.1016/S0140-6736(20)30183-5
12. Chen Z, Hu J, Zhang Z, Jiang S, Han S, Yan D, et al. Efficacy of hydroxychloroquine in patients with COVID-19: results of a randomized clinical trial. *medRxiv*. doi: 10.1101/2020.03.22.20040758. [Epub ahead of print].
13. COVID-19 Clinical Research Coalition. Global coalition to accelerate COVID-19 clinical research in resource-limited settings. *Lancet*. (2020) 395:1322–5. doi: 10.1016/S0140-6736(20)30798-4
14. The Lancet. COVID-19: protecting health-care workers. *Lancet*. (2020) 395:922. doi: 10.1016/S0140-6736(20)30644-9
15. Ranney ML, Griffeth V, Jha AK. Critical supply shortages — the need for ventilators and personal protective equipment during the Covid-19 pandemic. *N Engl J Med*. (2020) 382:e41. doi: 10.1056/NEJMp2006141
16. Goldman JD, Lye DCB, Hui DS, Marks KM, Bruno R, Montejano R, et al. Remdesivir for 5 or 10 days in patients with severe Covid-19. *N Engl J Med*. (2020). doi: 10.1056/NEJMoa2015301. [Epub ahead of print].
17. Spinner CD, Gottlieb RL, Criner G, Arribas López JR, Cattelan AM, Viladomiu AS, et al. Effect of remdesivir vs standard care on clinical status at 11 days in patients with moderate COVID-19: a randomized clinical trial. *JAMA*. (2020). doi: 10.1001/jama.2020.16349. [Epub ahead of print].
18. Wang Y, Zhang D, Du G, Du R, Zhao J, Jin Y, et al. Remdesivir in adults with severe COVID-19: a randomised, double-blind, placebo-controlled, multicentre trial. *Lancet*. (2020) 395:1569–78. doi: 10.1016/S0140-6736(20)31022-9
19. Skypper CP, Pastick KA, Engen NW, Bangiwala AS, Abassi M, Lofgren SM, et al. Hydroxychloroquine in nonhospitalized adults with early COVID-19: a randomized trial. *Ann Intern Med.* (2020) 16:M20–4207. doi: 10.7326/M20-4207

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Public Health

ORIGINAL RESEARCH
published: 24 September 2020
doi: 10.3389/fpubh.2020.563247

COMOKIT: A Modeling Kit to Understand, Analyze, and Compare the Impacts of Mitigation Policies Against the COVID-19 Epidemic at the Scale of a City

Benoit Gaudou[1,2,3]*, *Nghi Quang Huynh*[1,4], *Damien Philippon*[5], *Arthur Brugière*[1,3], *Kevin Chapuis*[1,6], *Patrick Taillandier*[7], *Pierre Larmande*[3,8] *and Alexis Drogoul*[1]*

[1] UMI 209, UMMISCO, IRD, Sorbonne Université, Bondy, France, [2] UMR 5505, IRIT, Université Toulouse 1 Capitole, Toulouse, France, [3] ICTLab, University of Science and Technology of Hanoi (USTH), Vietnam Academy of Science and Technology, Hanoi, Vietnam, [4] College of Information & Communication Technology (CICT), Can Tho University, Can Tho, Vietnam, [5] WHO Collaborating Centre for Infectious Disease Epidemiology and Control, School of Public Health, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong, China, [6] UMR 228, ESPACE-DEV, IRD, Montpellier, France, [7] UR 875, MIAT, INRAE, Toulouse University, Toulouse, France, [8] UMR 232, DIADE, IRD, University of Montpellier, Montpellier, France

Since its emergence in China, the COVID-19 pandemic has spread rapidly around the world. Faced with this unknown disease, public health authorities were forced to experiment, in a short period of time, with various combinations of interventions at different scales. However, as the pandemic progresses, there is an urgent need for tools and methodologies to quickly analyze the effectiveness of responses against COVID-19 in different communities and contexts. In this perspective, computer modeling appears to be an invaluable lever as it allows for the *in silico* exploration of a range of intervention strategies prior to the potential field implementation phase. More specifically, we argue that, in order to take into account important dimensions of policy actions, such as the heterogeneity of the individual response or the spatial aspect of containment strategies, the branch of computer modeling known as *agent-based modeling* is of immense interest. We present in this paper an agent-based modeling framework called COVID-19 Modeling Kit (COMOKIT), designed to be generic, scalable and thus portable in a variety of social and geographical contexts. COMOKIT combines models of person-to-person and environmental transmission, a model of individual epidemiological status evolution, an agenda-based 1-h time step model of human mobility, and an intervention model. It is designed to be modular and flexible enough to allow modelers and users to represent different strategies and study their impacts in multiple social, epidemiological or economic scenarios. Several large-scale experiments are analyzed in this paper and allow us to show the potentialities of COMOKIT in terms of analysis and comparison of the impacts of public health policies in a realistic case study.

Keywords: COVID-19, agent-based modeling (ABM), epidemiological modeling, GAMA platform, computer simulation (CS)

OPEN ACCESS

Edited by:
Zisis Kozlakidis, International Agency for Research on Cancer (IARC), France

Reviewed by:
Matteo Cinelli, Ca' Foscari University of Venice, Italy
Adeshina Adekunle, James Cook University, Australia
Mengmeng Li, Chinese Academy of Medical Sciences and Peking Union Medical College, China

***Correspondence:**
Benoit Gaudou
benoit.gaudou@gmail.com
Alexis Drogoul
alexis.drogoul@ird.fr

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Public Health

Received: *18 May 2020*
Accepted: *31 August 2020*
Published: *24 September 2020*

Citation:
Gaudou B, Huynh NQ, Philippon D, Brugière A, Chapuis K, Taillandier P, Larmande P and Drogoul A (2020) COMOKIT: A Modeling Kit to Understand, Analyze, and Compare the Impacts of Mitigation Policies Against the COVID-19 Epidemic at the Scale of a City. Front. Public Health 8:563247. doi: 10.3389/fpubh.2020.563247

1. INTRODUCTION

1.1. Context: The COVID-19 Pandemic

In December 2019, human infections by an unknown agent causing pneumonia were reported in Wuhan, China (1). The infectious pathogen, later known as SARS-COV-2, is a novel coronavirus responsible for causing the new COVID-19 disease.

While the first human cases appeared to be related to a seafood market, the following cases were not, indicating that SARS-COV-2 is capable of sustained human-to-human transmission (2). This preliminary investigation of the Wuhan outbreak in mid-January reported a baseline reproductive index (R0) of 2.2, meaning that the introduction of an infected individual into a fully susceptible population would result in an average of 2.2 additional infections. This strongly suggests that outbreaks could have grown exponentially if interventions and containment strategies had not been put in place early enough.

Given the initial lack of knowledge about the COVID-19 disease, the differences in preparedness, practices and cultural background of their populations, countries have naturally chosen different intervention policies to fight the pandemic. For instance, South Korea decided to move to massive drive-through virus testing programs after a fast increase of the number of infected cases (3), while France chose a late lockdown of the whole country [see (4) for an interesting overview of the strategies of 11 EU countries]. China imposed a lockdown to the most impacted city, Wuhan (followed by a lockdown of the entire province of Hubei) and implemented a strategy of contact tracing through the use of a smartphone application giving the exact location of an individual through time, allowing fast identification of contacts of an infected case (5). In Hong Kong, the fast implementation of border restrictions, isolations and quarantine, coupled with school closures and social distancing, has been shown really effective to reduce the transmission (6). Singapore initially chose to keep schools open, but performed health checks, reduced social gatherings, canceled large scale events, and traced contacts of infected cases, allowing the public to know the exact location of a known case once reported (7). Finally, Vietnam quickly chose to limit exchanges with China and applied very localized policies: for every identified infected individual, authorities tracked all the persons in contact with it and quarantined them. They also decided very early to lockdown full communes (e.g., Son Loi and Ha Loi in the province of Vinh Phuc) (8–10), an intervention similar to China but at a much smaller scale.

1.2. Proposal: An Agent-Based, Spatially Explicit, Modeling Kit

The wide range of possible interventions makes it extremely difficult to decide which ones are most appropriate in a given context. In this regard, computer modeling is an invaluable tool for exploring a range of intervention strategies *in silico* before the potential field implementation phase (11–13). It has been widely used, for example, to justify public health policies based on locking down entire populations (6, 14). However, while classical compartmentalized epidemiological models (15) or highly simplified individual-based models (16) seem to be relevant at the scale of an entire country, they are paradoxically not relevant at smaller scales, where it is of utmost importance to be able to accurately predict the impact of localized interventions. As a matter of fact, when an intervention is applied on a small population, the individual and social heterogeneities in terms of social or economic characteristics, medical profiles (17), spatial distribution (18), behaviors, opinion, or compliance to the public rules (19), are crucial factors to take into account in models. Moreover, among these features, some might remain constant (e.g., spatial distribution) but others can evolve during the intervention itself (e.g., compliance), making it difficult to approximate them with average values: models that only consider the evolution of the epidemic through the interactions between aggregated variables (representing compartments or other subsets of the population) are unable to represent these heterogeneities, let alone their evolution, and thus to use them for analysing, comparing, or even proposing possible interventions.

The urgent need of tools and methodologies that enable fast analysis of the effectiveness of the responses against COVID-19 across different communities and contexts, including small-scale ones, made us adopt an approach based on the design and simulation of agent-based computational models (20), where the profiles of people and households, their interactions, their evolution in time and space, are explicitly represented and serve as a basis for describing the dynamics of the epidemic. This is a "complex systems" perspective (21), where this dynamics is not only the result of a transmission mechanism, but also that of the non-linear interactions between actors with complex relationships and mechanisms across numerous levels of organization, which act and interact with each other and with their environment.

This has led us to design COMOKIT (COVID-19 MOdeling KIT) based on the agent-based modeling and simulation platform GAMA (22). As stated in Drogoul et al. (23), COMOKIT follows a set of principles:

- be as close as possible to public decision making by having the possibility to answer to concrete questions;
- be based on a detailed and realistic representation of space (public health policies are also predominantly spatial);
- rely on spatial and social data that can be collected easily and quickly;
- be generic, flexible, and applicable to possibly any case study;
- be trustable by relying on inner mechanisms that can be isolated and validated separately;
- be open and modular enough to support interdisciplinary cooperation;
- offer an easy access to large-scale experimentation and statistical validation by facilitating the exploration of its parameters;

This article is organized as follows. In section 2, we propose a rapid state of the art, which allows us to point out the limitations of existing models (whether mathematical or agent-based) in terms of decision support and realism in representing the impacts of interventions against COVID-19. Section 3 then presents the main structure and processes of the COMOKIT model, designed not only to overcome these limitations but also to provide a

basis from which more comprehensive models can be built. In section 4, we present a set of experiments carried out on COMOKIT with two ambitions: the first to show its dynamic characteristics (in terms of sensitivity to certain parameters, stochasticity and the need for replication), the second to show its potentialities in terms of studying and comparing the impact of public health policies in different scenarios. On the basis of these very encouraging initial results, section 5 concludes by listing some of the limitations of version V1.0 of the model and presenting its prospects for evolution and application to different contexts.

2. STATE OF THE ART

Several modeling studies have been undertaken at very early stages of the pandemic in order to study the impact of different policies against COVID-19 and to better prepare public health systems. Most of them relied on well-known mathematical models. As a matter of fact, at least in epidemiology, mathematical models are tools that can be developed very rapidly to answer a limited range of questions in critical and urgent situations. For example, such a model, using the meta-population of different cities represented by a Susceptible Exposed Infectious Recovered (SEIR) compartment model, was developed in less than a month to predict the spread of COVID-19 in a region or country and estimate the number of cases exported from Wuhan through human mobility and flights (24). This model was useful in showing whether and to what extent cases were likely to occur in areas other than Wuhan. Another mathematical model was used to represent the risk of virus introduction and the effectiveness of symptom screening in travelers (25). This probabilistic process model showed that, because of asymptomatic and pre-symptomatic infections, symptom screening alone was not sufficient to prevent the introduction of infected persons. Mathematical models have also been used to study control and non-pharmaceutical interventions in Europe, Wuhan and more abstract contexts (4, 26–28). For example, a model was designed taking into account the different contacts between the age groups represented in the SEIR compartments and examining the effect of control strategies implemented in Wuhan (27). Another model studied the effect of lockdown in European countries, assuming that the effect was the same regardless of the country of implementation, using a Bayesian approach (4). Health care capacity in the United States has also been studied using compartmentalized models representing individuals in the same age category in different states with different age-contact matrices (29). Finally, mathematical modeling was also applied prospectively to study the post-pandemic situation, examining seasonality and herd immunity (30, 31).

While mathematical models are particularly useful for rapid response and when there is a high degree of uncertainty in the different parameters, they also assume a certain homogeneity of individuals in a population, which can be a weakness when it comes to representing dynamics that rely heavily on individual aspects. While the use of age matrices in different compartmentalized models has countered this phenomenon, taking into account the fact that older populations appear to have a higher risk of developing a severe and more fatal form of the disease (4–6) while children are less likely to develop symptoms (32, 33), these models are still unable to take into account heterogeneities between individuals in terms of social relationships, behaviors, and attitudes toward the disease (34).

For example, intervention policies, such as lockdown are effective when everyone acts in accordance with policy statements. However, studies show that age groups may respond differently to containment, which may increase the risk of infection for that particular group (35, 36). This is particularly important because super-spreading events (infections of several people by one individual) have been reported in several locations (37). It is therefore essential to add complexity and heterogeneity in the models in terms of social relationships, spatialization, and individual characteristics. Although more complex to design and to explore (because of a generally more stochastic approach) than mathematical models, individual-based models have begun to be used to study COVID-19.

In Hellewell et al. (38), an individual branch process model is proposed to examine the possibility of preventing the introduction of the disease into a totally disease-free population by applying isolation and contact tracing. Interventions have also been studied in different contexts. For example, in Ferguson et al. (6), an ABM representing the population with different contact settings (school, work, home, etc.) for high-income countries has been designed to study the impact of different interventions to mitigate epidemics, including social distance, isolation of cases, quarantine and school closure. The model took into account spatialization but also individual characteristics to represent the risk profile, using the number of patients in intensive care units (ICUs), hospitalizations and deaths as indicators. However, the possibility of environmental transmission was not taken into account in the model. Indeed, several studies have shown that the virus can survive in the environment and on different types of surfaces (39, 40), possibly leading to environmental contamination and transmission, but also to nosocomial infections (41, 42). This type of transmission has already been reported in other coronaviruses, such as SARS and MERS (43, 44), and infections of several health care workers have also been reported (45). In addition, evidence of the viability of aerosolized virus transmission has also been provided (46). Another limitation of this model is that it does not account for hospitalizations, although it is known that some deaths are due to lack of hospital capacity. Finally, no information on recreational activities was represented, although bars, restaurants, nightclubs, cinemas and the like can be important contamination sites (47).

In Wang et al. (48), another model is presented, representing 2,000 people in four different states (susceptible-latent-infectious-removed) and examining a possible set of interventions, such as personal protection, isolation and quarantine, containment and social distance, and their cost-effectiveness after importation of infected cases. Again, intensive care and hospitalizations were used as indicators, but sociological aspects were not represented in this model. Transmission occurred in the community without taking into

account households, workplaces or other social gathering events known to facilitate the spread of the disease, and again, no environmental contamination was represented.

Modeling of pandemic transmission and control was also the objective of another study using ABM in Australia (19). In this model, interventions, such as school closures, travel bans, social distancing and case isolation were studied using an influenza-derived model representing a synthetic population of 24 million individuals with their own characteristics and social context. Nevertheless, no environmental contamination was represented due to the large scale of the model, which prevents the representation of buildings and other places. In addition, the model did not take into account the possibility of leisure activities, which can be explained by the fact that each stage corresponds to several hours (day and night periods). Finally, no dynamics concerning hospitalization capacity were represented.

Finally, another agent-based model, derived from influenza, was used in a study in Singapore (11), representing transmission during 12-h cycles for a set of buildings visited by infected persons. Again, school closure, quarantine and isolation of cases were studied, with an interesting aspect of the model being the focus on high-risk locations. However, as with the two models previously mentioned, no recreational activities were represented, as the temporal representation was done by day and night steps. In addition, environmental contamination was also not taken into account.

This rapid state of the art, far from being exhaustive due to the proliferation of more or less similar models, nevertheless makes it possible to highlight several limitations of existing models in terms of decision support:

- The limited and usually not flexible representation of individual activities does not allow these models to faithfully reproduce many social dynamics known to be at risk in terms of transmission: group leisure activities (karaoke, dance halls, restaurants, bars, etc.), groups at school or in companies, religious celebrations, etc.
- The often too large time step (day or half-day) cannot account for the shorter contacts or interactions that nevertheless constitute the bulk of our daily interactions. The resulting "averaging" effect erases any representation of the behavioral heterogeneity of individuals.
- In these models, individuals, even if their behaviors are different, are assumed not only to react in the same way to health authorities' injunctions, but also to do so in the same way regardless of when these injunctions are issued. However, a crucial point in the implementation of intervention policies is precisely to know how to anticipate the population's acceptance or rejection, and to be able to measure the effects of habituation, exasperation, or even revolt toward these policies.
- No environmental transmission is envisaged in any of these models, which raises the problem of their realism, especially when they take as a case study urban environments, where the opportunities for transmission through synthetic surfaces handled by many people (lifts, public transport, vending machines, handrails, counters, etc.) are legion.

COMOKIT has been primarily designed to meet these limitations. The following section presents version V1.0 of the model[1] in more detail.

3. MODEL

3.1. Overview of the Model

COMOKIT aims to simulate and compare the application of policies to mitigate the spread of COVID-19 at the scale of an urban area, with the disease being modeled at the individual scale. Its objective is to answer questions, such as: Is the containment of a neighborhood more effective than that of an entire village? Does school closure reduce transmission peaks? How does the wearing of masks affect the dynamics of the epidemic? What should be the ideal duration of containment? What proportion of the population should be allowed to engage in activities during a containment?

COMOKIT combines a sub-model of direct person-to-person transmission, a sub-model of environmental transmission through the built environment, a policy design model, and an agenda-based model of mobility and occupation of people at a rate of 1 h. A key point is that it allows the representation of heterogeneities in individual characteristics (gender, age, household), agendas (based on social structures, available services or age categories), social relations behaviors (e.g., compliance with policies), and response to COVID-19.

3.2. Description of the Model Entities

The central entity of the model is the *Individual* type (or species) of agents: it represents the individual inhabitants of the area under consideration with their individual characteristics (age, sex, occupational status) and their epidemiological status, whether they have been tested, and other individual epidemiological values (e.g., latent_time, infectious_time …more details in section 3.3.3). They carry out their daily activities (e.g., going to work, school, shopping, eating out, etc.) according to their personal agenda. This agenda is a set of generated activities that can be shared by several people (for example, going out to eat with friends), depending on the age and family status of the *Individual* agent. Attributes of *Individual* agents include their parents (their family, which in our model corresponds to the other *Individual* agents living in the same apartment in a *Building*), friends (with whom they can share activities), colleagues (co-workers or classmates), and their home, workplace, and school *Buildings*. An overview of the structure of the model is presented in the form of a UML class diagram in **Figure 1**.

Building agents are spatial entities where Individual agents can perform an Activity, which depends on the type of *Building*. Two special types of Buildings have been defined because they play an important role in the simulation: The Outside, which houses the activities performed by individuals outside the modeled area, and the Hospital, where sick Individual agents with critical symptoms

[1]The complete description of the COMOKIT model, using the classical O.D.D. protocol (49), is available at the address: https://comokit.org/ressources/ODD-COMOKIT_v1.pdf.

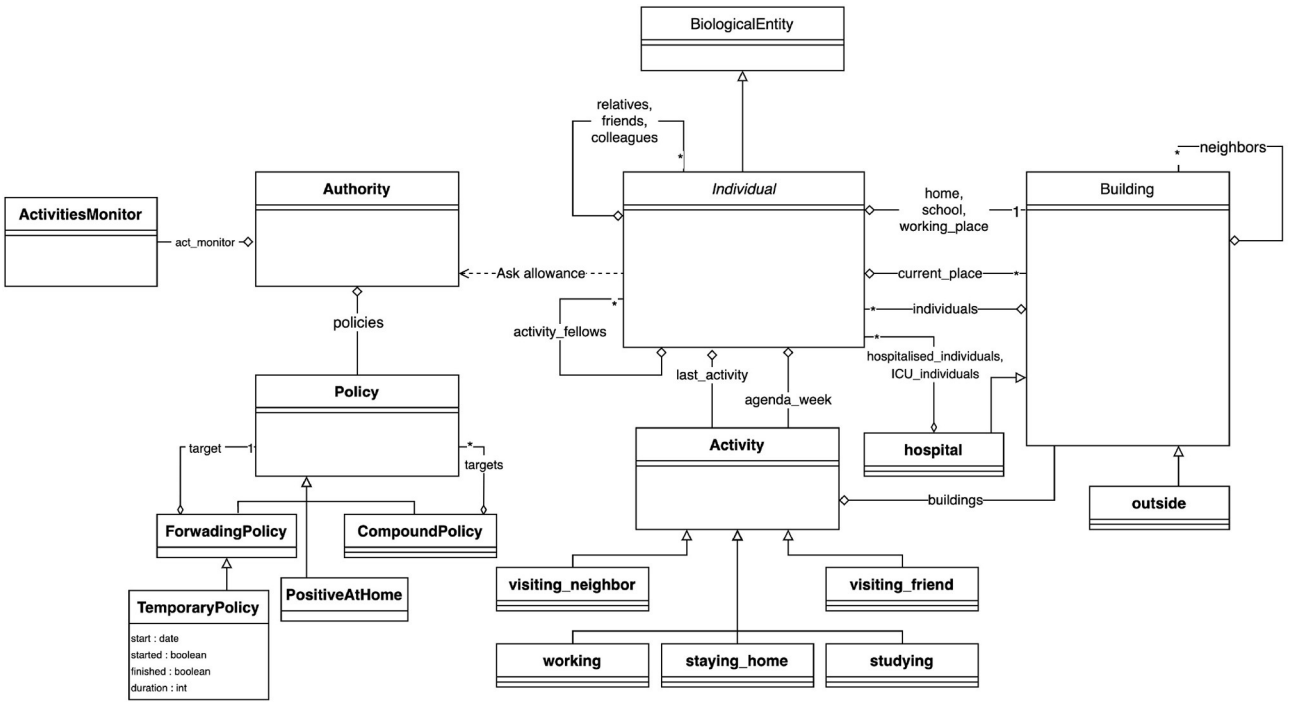


FIGURE 1 | Class diagram of the COMOKIT entities.

can be contained and treated. In order to take into account the possible transmission of the virus through the environment, all *Buildings* are equipped with a viral load, which can be used by the epidemiological sub-model (see section 3.3.3).

Individuals' hourly behaviors are determined by their agendas, which associate Activities with hours. Individuals have preferences for certain types of Activities that can be set according to their age and gender: for a leisure activity, a child may prefer to go to a play center while an older person may prefer to go to the cinema. The Building where Individuals carry out an Activity can be chosen at random (uniformly), as the closest, or according to a probability (negative function of distance and positive function of the area of the target place). COMOKIT also defines a number of specific Activities to represent some classical ones: visiting_a_neighbour, working, staying_at_home, studying, visiting_a_friend. Of course, custom activities can also be created from the generic Activity species.

In COMOKIT, particular attention is paid to policies that change the behavior of the population in order to reduce contact and thus infections between people: an Individual's ability to engage in a particular Activity is limited by the authorization of the Authority agent. Authorization to engage in specific activities depends on the Policy adopted and managed by this Authority. Examples of Policy include total containment, schools closure, working places closure …These Policies may be limited to a given area (using SpatialPolicy) or may be more or less tolerant (for example, containment may be complete or complete but for some people, or a certain percentage of the population, using PartialPolicy).

3.3. Description of the Model Processes

3.3.1. Initialization

A simulation is initialized by creating *Building* agents from shapefiles, *Authority* and *Policy* agents, and setting other parameters from data files. The *Individual* agents with their demographic attributes are created from a synthetic population generator [either an *ad hoc* generator coded in the model or by the Gen* generator using available data (50)]. Agendas are created using an *ad hoc* generator: they are composed by seven daily agendas depending on the *Individuals*' age and employment status: students and workers have an agenda composed of working days and leisure days (i.e., a day with activities different from working, learning or staying home); retired and unemployed *Individuals* have an agenda full of leisure days. *Individuals* that are too young have an empty agenda. The choice of activities outside of work and study will depend on the age and gender of the *Individual*. It is indeed possible to parameterize (through a CSV file) the fact that young people will, for example, favor leisure activities while elders will favor shopping activities. For each activity, a list of fellow *Individual* agents sharing the same activity can be defined to represent for example a group of friends or colleagues eating at the same table in a restaurant. Lastly, the simulation is initialized with N (a parameter) infected (but not yet infectious) *Individual* agents.

3.3.2. Process Overview and Scheduling

The dynamics of the model is entirely represented by three interconnected but nevertheless independent sub-models: **ESM**,

the epidemiological submodel (which combines infection, hospitalization and transmission processes), **ASM**, the activity submodel, and **PSM**, the policy submodel (which combines application and adoption processes). The simulation step is set to 1 h.

A simulation step starts by the evolution of the viral load in a building (it decreases over time, before disappearing). Then the Individual agents first evaluate whether they are infected. If they are, they may infect other Individuals and/or contaminate the current building in which they are located. Depending on their updated epidemic status, individuals will revise their behavior (e.g., wearing a mask) and execute their daily activities: they find the activity corresponding to the current hour, ask the Authority whether they are allowed to execute it and act in accordance. Finally, the Authority agent checks its current Policy and tries to apply it (e.g., executing a mass testing campaign).

3.3.3. ESM, the Epidemiological Submodel

As the virus is capable of surviving for long periods in the environment (39, 46), we consider two possible pathways of viral transmissions: either human-to-human transmission, through interactions between neighboring *Individual* agents, or, because of the potential persistence of the virus in the environment, through contacts between co-located *Individual* and *Building* agents, the latter of which being provided with a dynamic viral load (increased by the long-term co-location of infectious individuals, and decreasing according to some decay).

In our model, the disease-related state of the Individual agents follows a slightly modified SEIR model (15) (**Figure 2**). First, we assume that the whole population starts the simulation in the **Susceptible** state (**S**): as this is an emergent disease, nobody is immunized. When an *Individual* is in contact with an infectious *Individual* or located in an infected *Building*, it can become infected and move to the **Latent** state (**L**) (a renaming of the traditional *Exposed* compartment), depending on the success of the transmission, defined by the probability for one *Individual* at a given step to be infected by an infectious *Individual* in the same *Building*, or by a *Building* with a positive viral load.

Once the latent period is expired, an *Individual* transitions to one out of three possible infectious states (whereas the traditional SEIR model contains only a single one): it can become **asymptomatic** (IA), **pre-symptomatic** (IP) or **symptomatic** (IS). If the *serial interval* value is negative, it becomes **pre-symptomatic** for a short time, equal to the absolute value of the *serial interval*, before transitioning to the **symptomatic state**. The *Individual* remains in these states during the *serial interval* (for **pre-symptomatic** ones) or the *infectious period* for **symptomatic** and **asymptomatic** ones. Finally, we consider that **asymptomatic** and **pre-symptomatic** Individuals share the same *transmission rate*, i.e., the chance of infecting a neighboring susceptible *Individual*, while **symptomatic** agents have a much higher one.

After the *infectious period*, *Individual* agents become **Removed** (**R**): they are not infectious anymore and fall into one out of two sub-compartments, **Recovered** (**RR**) or **Dead** (**RD**).

During their infectious period, symptomatic individuals can go through different clinical states: not needing hospitalization (**NH**), needing hospitalization (**HN**) and needing ICU (**HI**). Previous **asymptomatic** agents (la) become directly **Recovered**, as we assume that they cannot die from COVID-19 without showing symptoms, whereas symptomatic ones have a probability to recover or die (**Figure 3**). This probability depends on the (given) severity of the disease for the age category of the agent and the care it has been provided with (i.e., hospitalization and ICU). We consider that *Individuals* needing intensive care will become **Dead** if they do not get it. On the contrary, symptomatic *Individuals* that do not need intensive care (i.e., not needing hospitalization or needing hospitalization without intensive care treatment) become **Recovered**.

It is important to note that **ESM**, despite the fact that it is a more detailed model than most of those used in agent-based models (19, 48), makes certain assumptions, some of which are shared with other epidemiological models because of a lack of knowledge about the disease, others because we assume that they have no influence on the model itself.

1. Effective contact rate
   (a) Presymptomatic and asymptomatic individuals share the same contact rate
   (b) The contact rate does not differ during the infectious period.
   (c) Masks do not deliver any protection, but rather reduce the effective contact rate of an infectious individual and its viral release in the environment.
2. Environmental transmission
   (a) *Individuals* can be infected by a contaminated environment, and for a maximal viral contamination in one building, the effective contact rate is the same as one infectious *Individual*.
   (b) The viral release of an *Individual* in its environment (in our model, in *Buildings*) is the same for all infectious *Individuals*
3. Homogeneity of the population
   (a) The sex of individuals does not have any impact on the epidemiological model.
   (b) The age of individuals does not have any impact on the incubation period, the proportion of asymptomatic cases, and the effective contact rate for human to human and environmental transmission
   (c) Asymptomatic and symptomatic individuals share the same infectious period distribution
4. Recovery and death
   (a) Recovered Individuals are totally immunized against the infection.
   (b) Infection can lead to death only for *Individuals* expressing a need for intensive care.

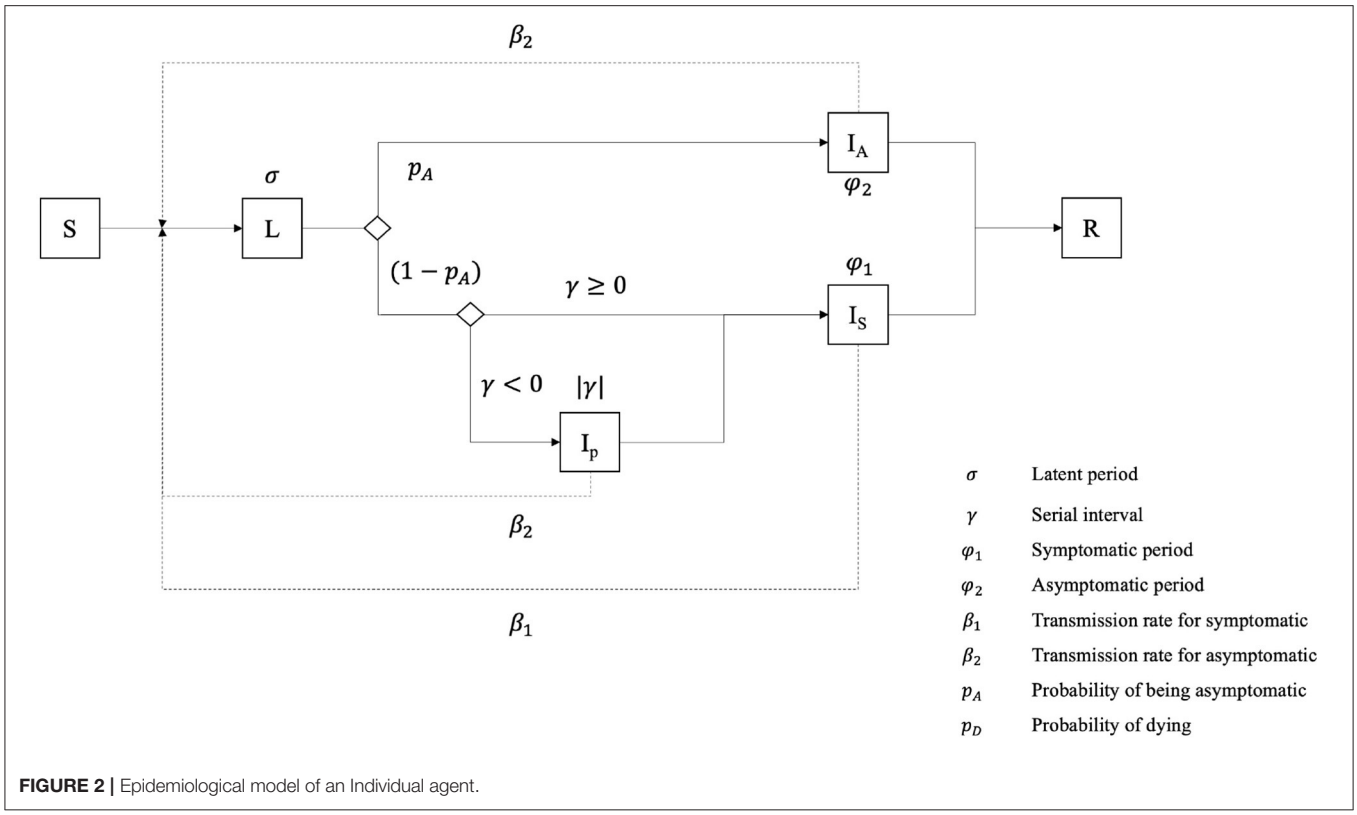


FIGURE 2 | Epidemiological model of an Individual agent.

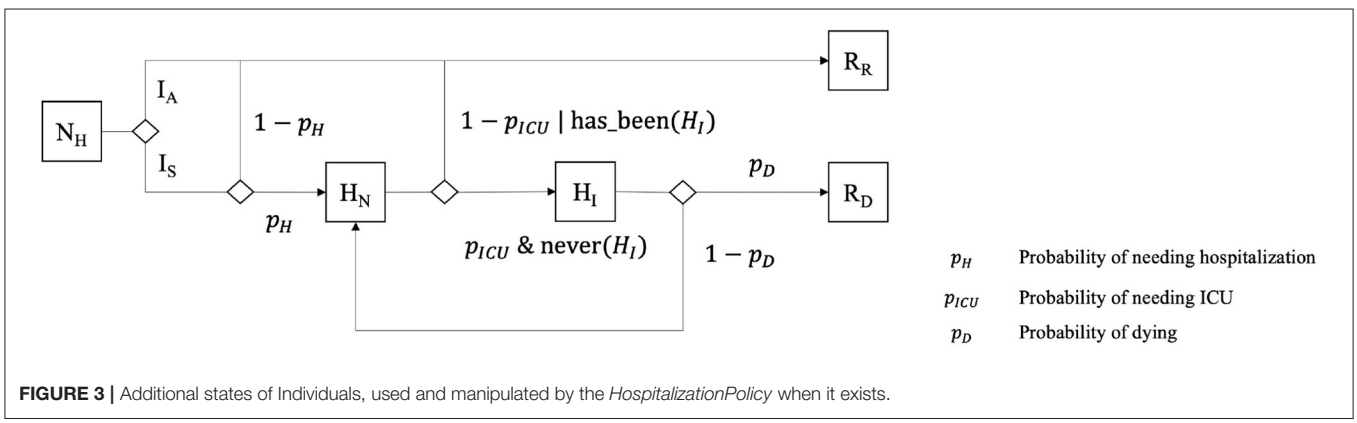


FIGURE 3 | Additional states of Individuals, used and manipulated by the *HospitalizationPolicy* when it exists.

(c) Testing is performed only for virus isolation, not antibodies, therefore recovered people are not considered positive.

3.3.4. ASM, the Activities Submodel

The *Individual* agents in COMOKIT are an extremely simplified representation of their actual counterparts; their daily activities are ultimately only the dynamic support of their role as disease spreaders. These activities, as discussed in section 3.3.3, are organized in the form of a weekly agenda that can distinguish between days off and days worked, and provides an hour-by-hour activity for all the agents. Once the weekly and daily agendas are created, at each simulation step, and unless they have already been enrolled in a collective *Activity*, *Individual* agents obtain the *Activity* corresponding to the current day and time, request the authorization from the *Authority* agent to perform it, and find a nearby building associated with this specific *Activity*. *Individual* agents can also enroll certain agents to participate in the *Activity* (e.g., colleagues, friends …) who are expected to have a closer relationship, and therefore have a higher probability of being infected. Since we have set the time step at 1 h, we decided not to represent the movement itself from one place to another : *Individuals* are translated directly from their current location to the building chosen to perform their new *Activity*.

This last choice may appear to be a limitation (or at least a somewhat too restrictive assumption), but it is consistent with the

scale at which we model the disease and the control policy. Public transport, which represents one of the main risks of transmission in large conurbations or on national and international scales, is not used in small or medium-sized towns, which are the current target of COMOKIT.

3.3.5. PSM, the Policy Submodel

The *Authority* is responsible for implementing one or more mitigation policies that may impact the simulation in two ways: at each step, on one hand, the *Authority* may proactively perform certain actions, for example by conducting a given number of tests on the population, and on the other hand, each *Individual* agent asks the *Authority* whether it is authorized to perform a given activity.

We have chosen a modular approach to defining policies: a general policy is based on a small set of specialized, concrete policies (e.g., the *DetectionPolicy* that authorizes all activities, but performs tests at each step, or the *ActivitiesListingPolicy* that limits activities within a given set of authorized activities) that are composed using the composite (implemented by *CompoundPolicy*) and nesting (by *ForwardingPolicy*) design patterns:

- *CompoundPolicy* is a policy composed of a list of other policies. It applies the policies listed in order and allows an activity for a given individual if and only if it is allowed by all policies.
- *ForwardingPolicy* is a policy that embeds another policy and can change its enablement dynamically (for example, the specialized *SpatialPolicy* restricts the application of its target policy in a given geographical space, while *TemporaryPolicy* does it within a limited period of time).

Among the different policies delivered with the standard version of COMOKIT is the one that explicitly links to the epidemiological sub-model **ESM** (without being necessary for its operation). This is the *HospitalizationPolicy*, which depends on the existence of at least one hospital *Building* in the dataset, and which takes care of the *Individuals* that need to be hospitalized after a certain period of time following symptom onset, given by a distribution, and remain hospitalized until they are recovered or dead. Hospitalized *Individuals* are considered **Recovered** after having tested negative for a given number of consecutive days, and not showing symptoms (i.e., not being in the **Symptomatic** state).

The availability of these policies and the ease with which they can be combined make it possible to represent complex and realistic public policies. For instance, a "realistic lockdown" experiment was created to test the impact of a 60-days lockdown policy, in which positive individuals are not allowed to travel, others are only allowed to stay at home or shop, and only 10% of the total population is allowed to work. The policy of the *Authority* in this experiment is therefore constructed as a *TemporaryPolicy*, limiting the application of a *CompoundPolicy* within a 60-days period. This nested composite policy was composed of:

- A policy applying a given number of tests at each step of the simulation (this policy allows any activity).
- A policy prohibiting any activity other than shopping and staying home, nested in another policy that limits its application to 90% of the population (the remaining 10% are free to engage in any activity).
- A policy prohibiting any activity for those who have tested positive.
- The hospitalization policy described above.

3.4. Input Data

All input data files used to initialize a COMOKIT simulation are summarized in **Table 1**. In addition to the geographic data (buildings.shp, boundary.shp, and satellite.png), the files describe either the synthetic population of Individuals generated by an external tool or the parameters of the generators integrated in COMOKIT.

3.4.1. Spatial Data

The initialization of the spatial environment of the model requires one main input: a shapefile describing the buildings of the studied area. This shapefile must obligatorily contain two attributes: the type of building, which will be used in the definition of activities (each type of activity will be linked to one or more types of buildings), and the number of apartments per building, which is used to locate the households inside (one household per apartment). COMOKIT provides a spatial data generation tool, allowing, from a spatial boundary given as a shapefile, to download existing OSM[2] data of the area and put it in the right format so that it can be directly used in the simulations. The existing tool also allows the vectorization of images (e.g., GoogleMap) to enrich the OSM data.

3.4.2. Demographic Data

The simulation initialization can use a CSV file describing the synthetic population, where each line (also called *record*) corresponds to a unique individual with age, gender, household identifier and employment status. The Gen* library (50) can be used to generate such a population file from an IPUMS[3] open-access population sample file and the marginal distributions of the demographic attributes available on the given case study. The generation of this synthetic population follows the combinatorial optimization approach described in Williamson et al. (51). Among the various algorithms available, we chose a simple random draw in order to fit the actual population sample to a known aggregate distribution of attributes. This algorithm begins with a random population (containing the desired number of individuals) composed of households uniformly selected from the sample; we then exchange n records of the synthetic population with records drawn from the sample. This operation is repeated until either a minimum matching is obtained or a maximum number of iterations has been performed. In the different experiments we made, we found that the algorithms

[2]OSM stands for OpenStreetMap. The data have been accessed and retrieved from the website: https://www.openstreetmap.org/, using the provided Application programming interface (API).
[3] https://ipums.org/

TABLE 1 | Overview of the dataset.

Data file	Data type	Description	Source
Buildings.shp	GIS shapefile	Geometries of buildings, with their type and number of flats as attributes	OpenStreetMap, Google Maps, and hand digitalization from Google satellite image. For Ben Tre, the initial data come from the Land Use map (produced by the DONRE* in 2010)
Population.csv	CSV tabular file	The synthetic population generated from a sample using the Gen* library. Each line corresponds to a single individual with age, sex, and household id	https://international.ipums.org/international/ https://www.gso.gov.vn/default_en.aspx?tabid=774
Population parameter.csv	CSV tabular file	The set of parameters to define the population of Individuals	See O.D.D. description for more details
Activity parameter.csv	CSV tabular file	The set of parameters to define the activity of Individual	See O.D.D. description for more details
Activity type weights.csv	CSV tabular file	According to the age (interval) and sex, the weight of the different activities	See O.D.D. description for more details
Building type weights.csv	CSV tabular file	According to the age (interval) and sex, the weight of the building type	See O.D.D. description for more details
Epidemiological Parameters.csv	CSV tabular file	The set of epidemic parameters for the COVID-19	Various sources from the literature (see O.D.D. description for more details)

**DONRE stands for Department Of Natural Resources and Environment. This is a department of the Vietnamese Ministry Of Natural Resources and Environment.*

performed well with n equal to 5% of the population size and a maximum number of iterations equal to 100.

The obtained population contains only demographic variables. These are supplemented by built-in COMOKIT generators for location, social network, and agenda.

3.4.3. Epidemiological Data

The epidemiological parameter file is a table of parameters. For each parameter, the following values are provided: (i) the name of the parameter, (ii) the lower limit of the age category, (iii) whether the value of the parameter is given or whether it is to be chosen from a given probability distribution (and in this case the distribution considered), (iv) its value (if of a given value type) or the first parameter of the distribution, and (v) the second parameter (of the distribution). These data contain in particular the parameters that will make it possible to specify, in different case studies, the human-to-human transmission (within households, during activities) and environmental transmission processes.

3.5. Outputs

Much of the rapid assessment of a model's relevance depends on its ability to display results in a way understandable by its designers, programmers and users. When COMOKIT is used in the form of a dashboard or a demonstrator, the user interface of the simulations that each experiment runs can be completely defined and specialized according to the needs of its users. As the model was primarily designed to evaluate and compare policies, most experiments run several simulations in parallel with different parameter values. The user interface then contains a display of the spatial evolution of the disease for each parameter value and a graph plotting the evolution of the number of infected individuals over time. **Figure 4** shows an example of such an interface considering five different proportions of unconfined individuals. It is also possible to display decreases in activity for different types of activities (compared to a baseline where no policy would be applied). Many other visualizations are possible, both in 2D and 3D, using the declarative approach proposed by GAMA. Some of them are provided in the model as a base, but can be enriched according to the needs of the users in order to compose real dashboards.

4. EXPERIMENTS

In order to illustrate how COMOKIT can be used, we conducted a series of experiments for Son Loi Commune in Vinh Phuc Province, Vietnam. Son Loi is a rural commune of about 10,600 inhabitants and has 3,000 buildings of different types (houses, schools, temples, administrative buildings, industries...). Returning from a business trip to China, the first two cases were identified on January 17, 2020 (9). After the identification of nine other cases (on the 13th of February), the provincial authorities decided to lockdown the entire commune: the inhabitants were advised to stay at home and could not leave the commune; their state of health was checked daily and the authorities organized the supply of food and masks. After 18 days with no new cases identified, the lockdown was lifted on the 2nd of March.

To initialize the simulations, we first obtained spatial data on the buildings of the commune from the buildings present in Google Map and Bing data. The population input data file was generated using the Gen* library to produce a set of individuals grouped into households. We then used an approach based on combinatorial optimization to find a trade-off between maintaining the consistency of the microdata sample at the household and individual levels, while trying to match the census marginals (e.g., number of men/women, frequency of age category). In our case study, we used the IPUMS sample of individuals in households available for the

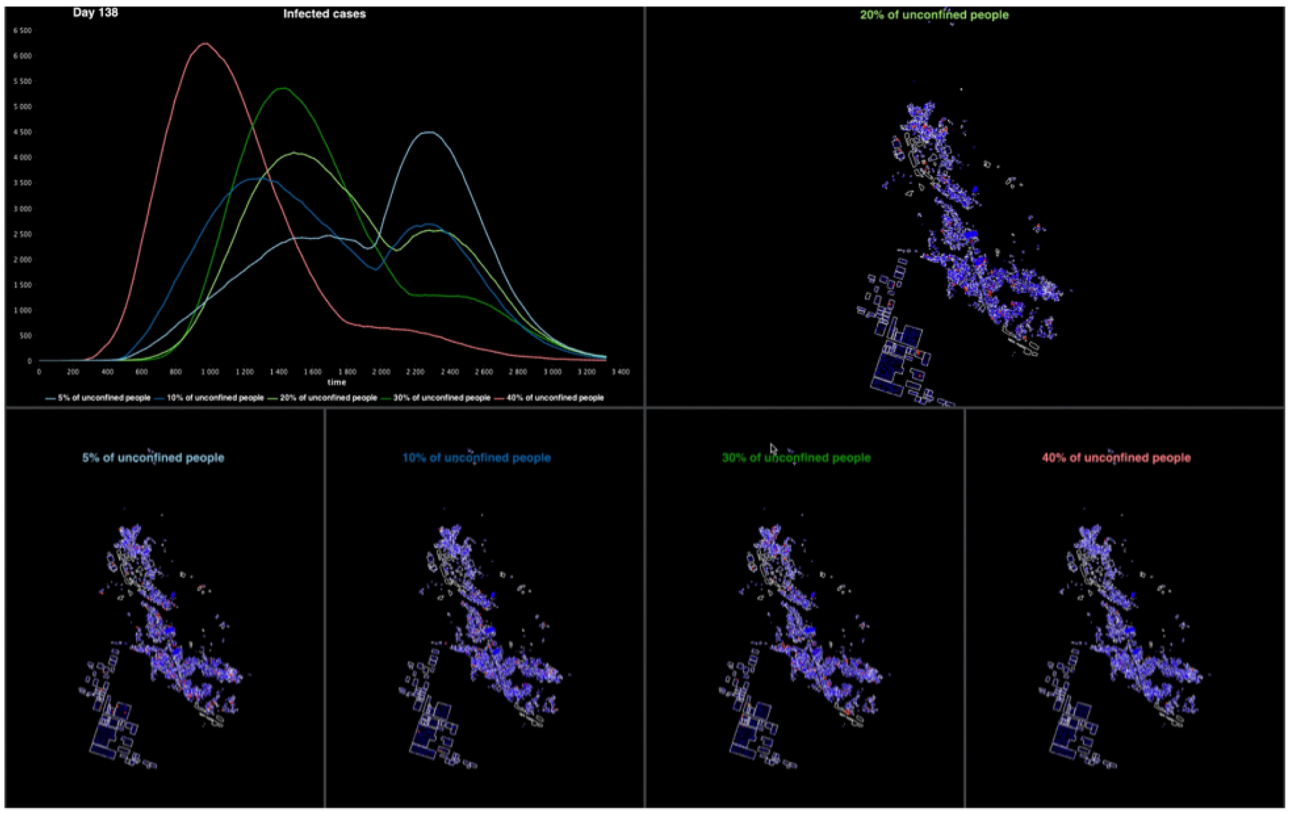


FIGURE 4 | Example of the graphical interface of a COMOKIT experiment on Son Loi commune. The experiment compares five simulations with different numbers of unconfined people.

whole Vietnam in 2014 (15% of the total population) with age, sex, household identification and employment status. Then, we randomly selected households with corresponding individuals in the sample to match the age and sex distribution at the individual level that we found in the 2019 Vietnamese census for Son Loi. The indicator chosen to assess the quality of the population is a normalized Total Absolute Error (TAE)[4]. The normalized TAE of the best synthetic population (that is used in the experiments below) is 0.1. This means that in the best generated population, when considering the distributions in each age and sex category, the number of individuals in the synthetic population differs on average by 10% from the aggregate census count.

As far as epidemiological parameters are concerned, most of them come from the literature. A preliminary calibration step is nevertheless necessary to make the disease transmission rate matching with data available on the considered case study: the "Successful_contact_rate_human" (the main parameter impacting the transmission between human beings) is computed given the R0 of the epidemic in the considered area and the average number of contacts between people in the simulation[5].

We used the default value for all the other parameters (see the O.D.D. description of the COMOKIT model for the complete set of values, c.f. section 3).

4.1. Stochasticity Sensitivity Analysis

In a first experiment, we analyze the impact of the randomness of the simulations on the results and in particular on the dynamics of the epidemiological status of *Individuals*. The main objective is to find a threshold value of replications beyond which an increase in the number of replications would not imply a significant marginal decrease of the difference between the results. To do this, we compare the global incidence (defined here as the number of new infected individuals per time step), and the number of *Individuals* recovered and dead, between replications of the simulation. Incidence dynamics are not expected to be smooth since the number of new infections depends on the contacts between *Individual* agents, and those do not have much contact when staying at home during the night. We undertake this exploration with the simplest possible scenario, i.e., a free spread of the disease without containment and two infected

[4]Consider the distribution tables of age and sex attributes in the real population and in the synthetic population. The TAE is "the sum of absolute differences between cells" in these two tables (51), and the normalized TAE is the TAE divided by twice the population size. This normalized TAE represents the rate of individuals with at least one incorrect value in a category.

[5]The method is detailed on the COMOKIT website: https://comokit.org/docs/parameterize#calibration-of-the-transmission-rate-value-beta.

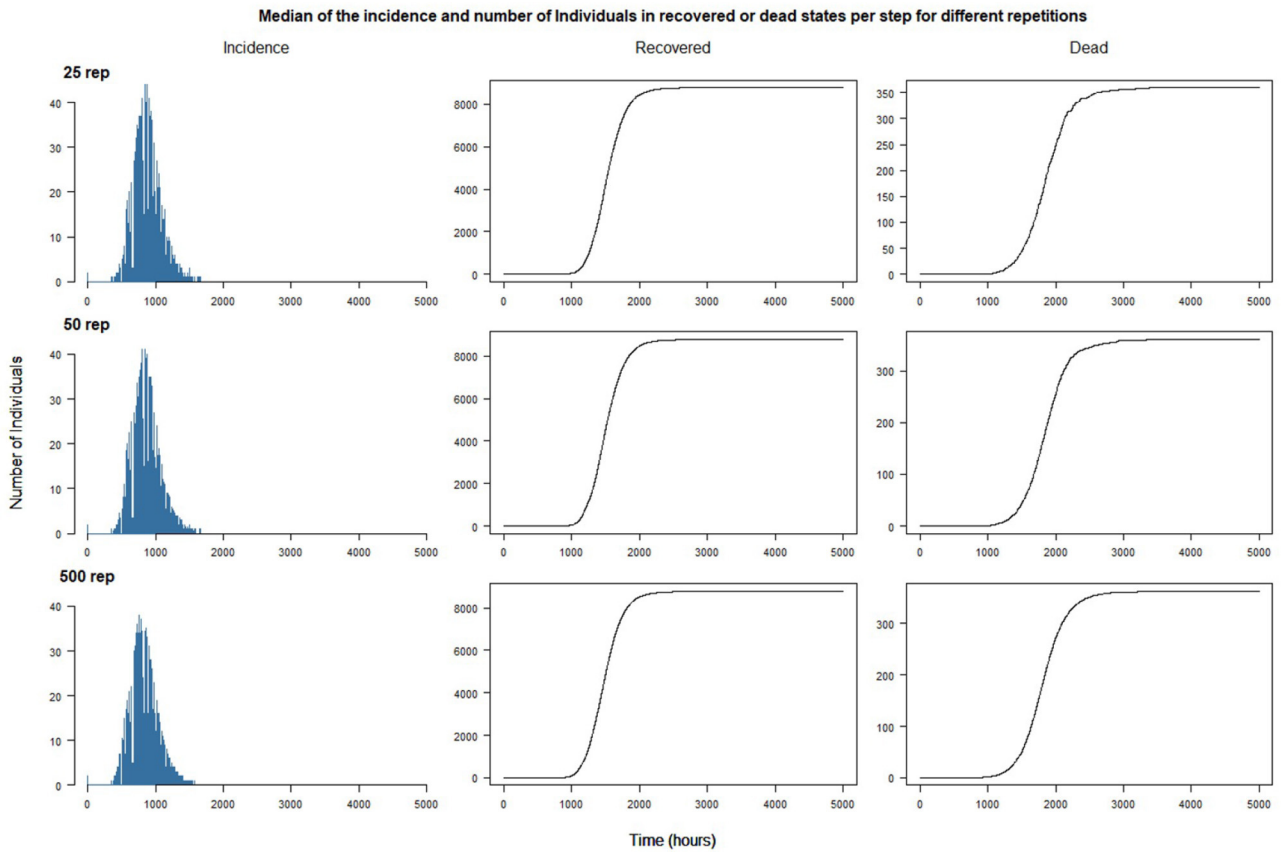


FIGURE 5 | Median of the incidence and number of individuals in recovered or dead states per step for 25, 50, and 500 different repetitions.

individuals at the beginning of the simulation. We perform 500 replications of such a simulation and compare the variability of the results for the first 25, 50, 100, 250, and 500 replications.

In **Figure 5**, we plot the median value (over the replications), by time steps, of the incidence (first column), the number of individuals recovered (middle column) and dead (last column). The shapes are as expected for a SEIR-like epidemiological model of the disease: the incidence increases exponentially until a peak before decreasing to 0, and the number of Recovered and Dead Individuals increase until a time step where they become constant.

The results suggest that increasing the number of replications beyond 25 does not have a great impact on the aggregate trend of the simulated epidemic: the curves soften as the number of replications increases, but the patterns remain the same. One of the reasons is certainly the absence of interventions outside the introduction of the first two cases: the dynamics of propagation is ultimately only marginally influenced by the usual activities of the agents. On the other hand, we can expect the simulations to show quantitatively and qualitatively different results, or greater variability, when interventions will be introduced (see next section).

Figure 6 plots the simulation steps for the maximum of incidence and the steps to reach the maximum of the number of Dead and Recovered Individuals: it shows the median (black line), the second and third quartiles (the box) and the minimum and maximum peak cycle (whiskers) excluding *outliers* (simulation results that differ from the median by more than 1.5 times the IQR). We can observe that most of the simulations show a near peak cycle, between 500 and 1,500 for incidence, 3,000 and 4,500 for recovered individuals and 2,500–4,000 for deaths: this shows that the number of replications does not have a large impact on the aggregate outcome. However, after more than 100 replications, we have observed some simulations that show a very contrasted behavior: for example, when performing 500 replications, three simulations have their maximum number of agents recovered at less than 1,000 cycles, which means that the epidemic is not occurring or at least that the spread of the epidemic has been rapid and less impacting. The probability of "extreme" outcomes occurring (e.g., a long duration or complete absence of epidemic spread) is obviously positively correlated with the number of replications.

For the policy impact study presented in the following section, we decided to set the number of replicates at 50 in order to minimize the computation time required while trying to maintain realistic statistical properties, in particular the occurrence of extreme outcomes.

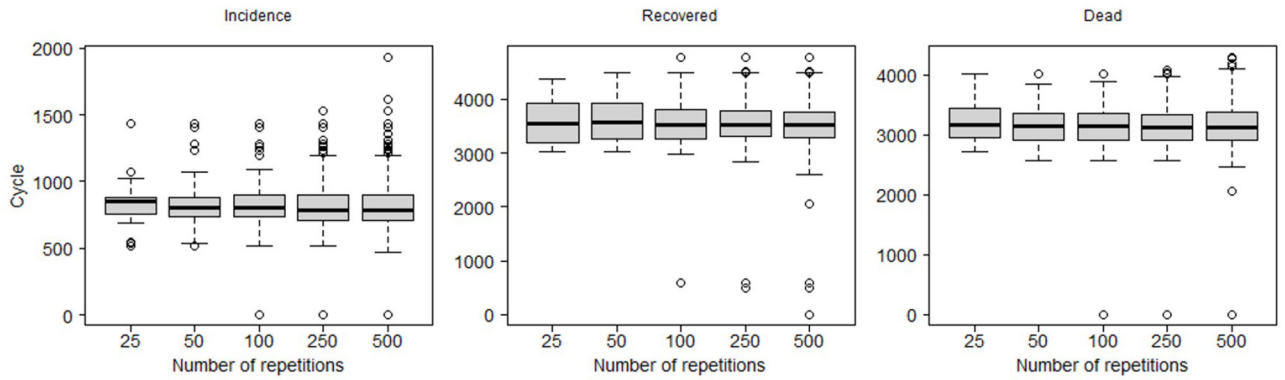


FIGURE 6 | Whiskers plots and minimum/maximum excluding 1.5 IQR outliers of the simulation step of the maximum of the incidence, and the minimum step to reach the maximum of the cumulative number of recovered and dead individuals per step for different number of repetitions.

4.2. Comparison of Policies

In this section, we illustrate the possibilities of COMOKIT, on the same case study (Son Loi, Vietnam), for comparing the impacts of policies and analyzing their performance against a reference scenario where the virus would have spread freely. The simulations presented here are limited in number, as the objective is not to provide exhaustive results, for which an overall sensitivity study would have been necessary, and which would in any case make no sense for this particular case study, but to show what can be achieved with the simulator.

4.2.1. Impact of Wearing Masks

The objective of this experiment is to evaluate the impact of wearing masks on the spread of the epidemic. While masks are still not recommended for the general population by the WHO and there is scientific debate on their use (52), a study has shown the ability of surgical masks to prevent the exhalation of respiratory viruses (53). In addition, asymptomatic and presymptomatic COVID-19 infections have been reported in different locations (54–57), and are suspected to play an important role in the persistence of epidemics (58). Therefore, the use of masks by the population could reduce the impact of presymptomatic and asymptomatic carriers by preventing them from releasing aerosols when they are not yet symptomatic, or droplets when they sneeze (not necessarily related to the disease). Due to past events related to respiratory diseases, such as SARS and influenza, people in Asian countries have been extremely cautious, wearing masks from the onset of the COVID-19 epidemic as a hygienic practice, even when people did not show any symptoms (52).

We therefore sought to assess the impact of the proportion of people wearing masks on the total incidence, the number of people recovered and the number of deaths. A comprehensive experiment exploring one parameter of the simulation (the proportion of individuals wearing a mask, taking a value between 0 and 1 and a step size of 0.25), was then launched. For each value of this parameter, we ran 50 replications.

In **Figure 7**, even if wearing a mask does not help reduce the total number of infections or deaths (because it only influences disease transmission), it is found that the use of masks helps to flatten the incidence curve. Therefore, recommending the use of face masks would avoid overloading hospitals and intensive care units in our model as much as possible. The most important change in the dynamics of the incidence curve was achieved with a probability of wearing face masks of 0.75 (and above), which avoided the sudden increase in cases that was still noticeable with a probability of wearing face masks of 0.5. Since the policy applied was only to wear masks, no symptomatic individuals were admitted to hospital. Therefore, neither hospital overload nor the benefits of being admitted were simulated. The number of deaths did not change, but the reduction in the number of infected persons should avoid exceeding hospital capacity as much as possible.

4.2.2. Impact of the Duration of the Lockdown on the Epidemic Peak

Faced with a pandemic without specific treatment or vaccine, public health services rarely have any choice but to choose policies to limit transmission and "flatten the curve" of incidence in the population. Depending on the country and region, there is a range of measures from soft social distancing, such as wearing masks in public spaces, avoiding congested areas or maintaining a distance of 1 m from other public transport users, to blanket travel restrictions, forced quarantine, total containment and technological monitoring (59). The positive effects of such actions on the number of hospitalizations, intensive care admissions, deaths or on the reproduction number have been demonstrated in different contexts, such as France (16, 60), Wuhan in China (61) or Italy (62). While complete lockdown appears as one of the best ways to mitigate the spread of an epidemic, it raises serious concerns related to economic

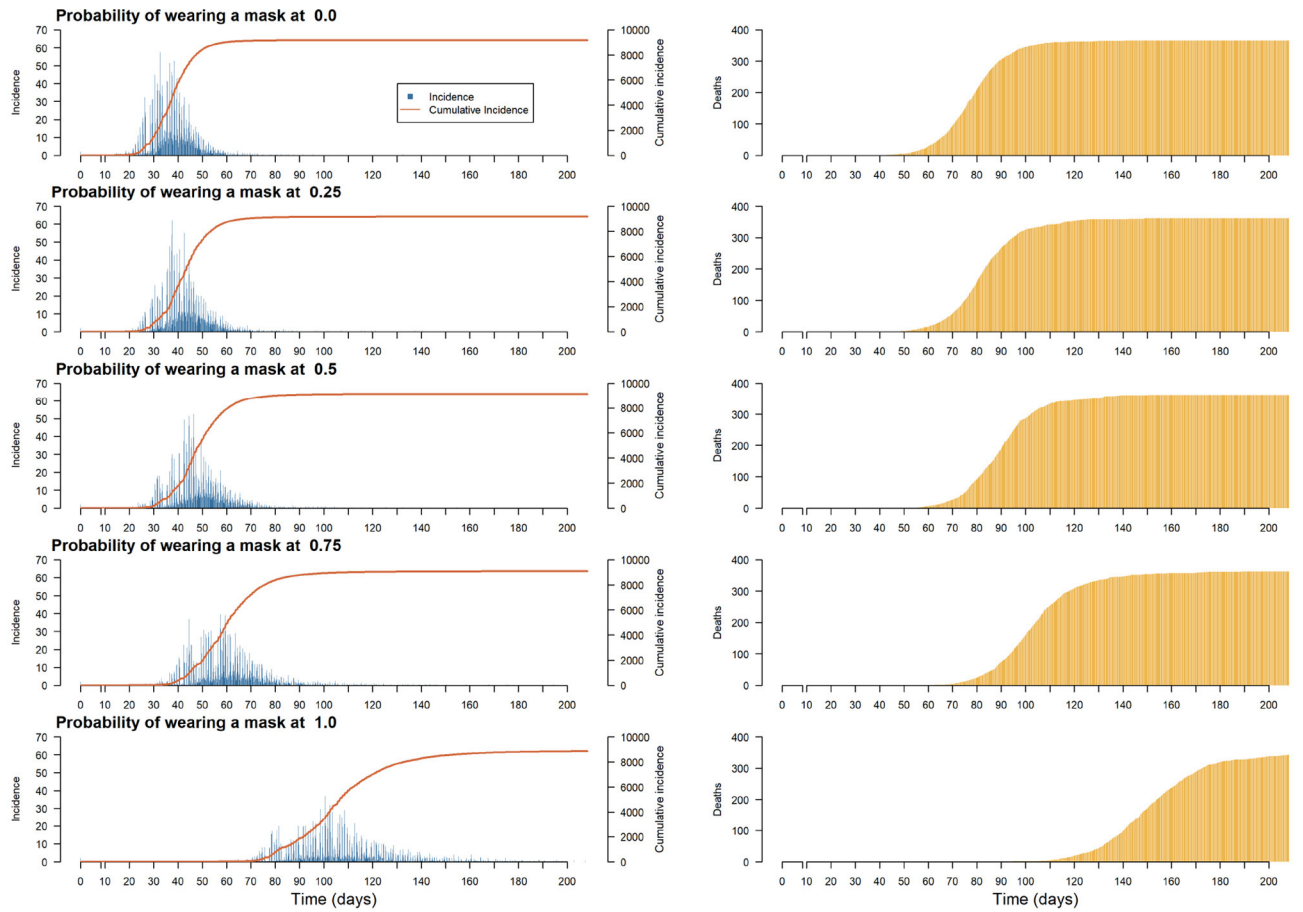


FIGURE 7 | Plots of the median of the incidence and cumulative incidence and deaths on the population per step for different proportions of the population wearing a mask.

(63) and socio-psychological (64) outcomes and also questions about the duration of its effectiveness (65) or the consequences of a partial or total lifting of restrictions (16).

The aim of this experiment is to evaluate the impact of the duration of a complete lockdown (i.e., when all the activities are forbidden) on the incidence, the number of recovered and dead Individuals. Simulations are launched with a simulation parameter encoding the duration of the lockdown taking values among 0 (no lockdown), 15, 30, 45, 60, and 90 days. More specifically, we observe how lockdown duration modifies the magnitude (e.g., lower or flatten) and time frame (e.g., happen fast or last long) of the epidemic peak. All the simulations are initialized with two new infected Individuals chosen randomly in the population. The complete lock-down policy is applied at the initial state of the simulation. The case study is also a simplified situation as no infected Individuals, external to the commune, can enter in the commune during the simulation.

In **Figure 8**, we have plotted the incidence, cumulative incidence and deaths in the population, per step, for different lockup durations. First of all, we can see that it is not necessary to continue confinement after 60 days, as this is enough time to let the disease disappear. For shorter durations, preliminary results show that a peak in the number of infected individuals cannot be avoided, although confinement for between 15 and 45 days tends to delay the peak (by giving the health services more time to prepare) and flatten the curve (by avoiding overloading hospitals).

4.2.3. Comparison of Realistic Policies

The objective of the last experiment is to compare the impact on the same case study of three realistic public health policies:

- *A combination of policies similar to that used in South Korea*: mass testing (in the model: more than 900 tests per day) with home quarantine for households with confirmed cases. South Korea is recognized as one of the countries with the most effective mitigation strategies implemented: according to the UNDP (3), it was one of the first countries to implement mass test programs (between 15 and 20,000 tests per day) with home quarantine guidelines for confirmed cases. The South Korean government's rapid and organized response has produced excellent results in freezing the early spread of the epidemic.
- *A combination of policies similar to the one used in France*: few tests (in the model: less than 200 per day) and, from 1%

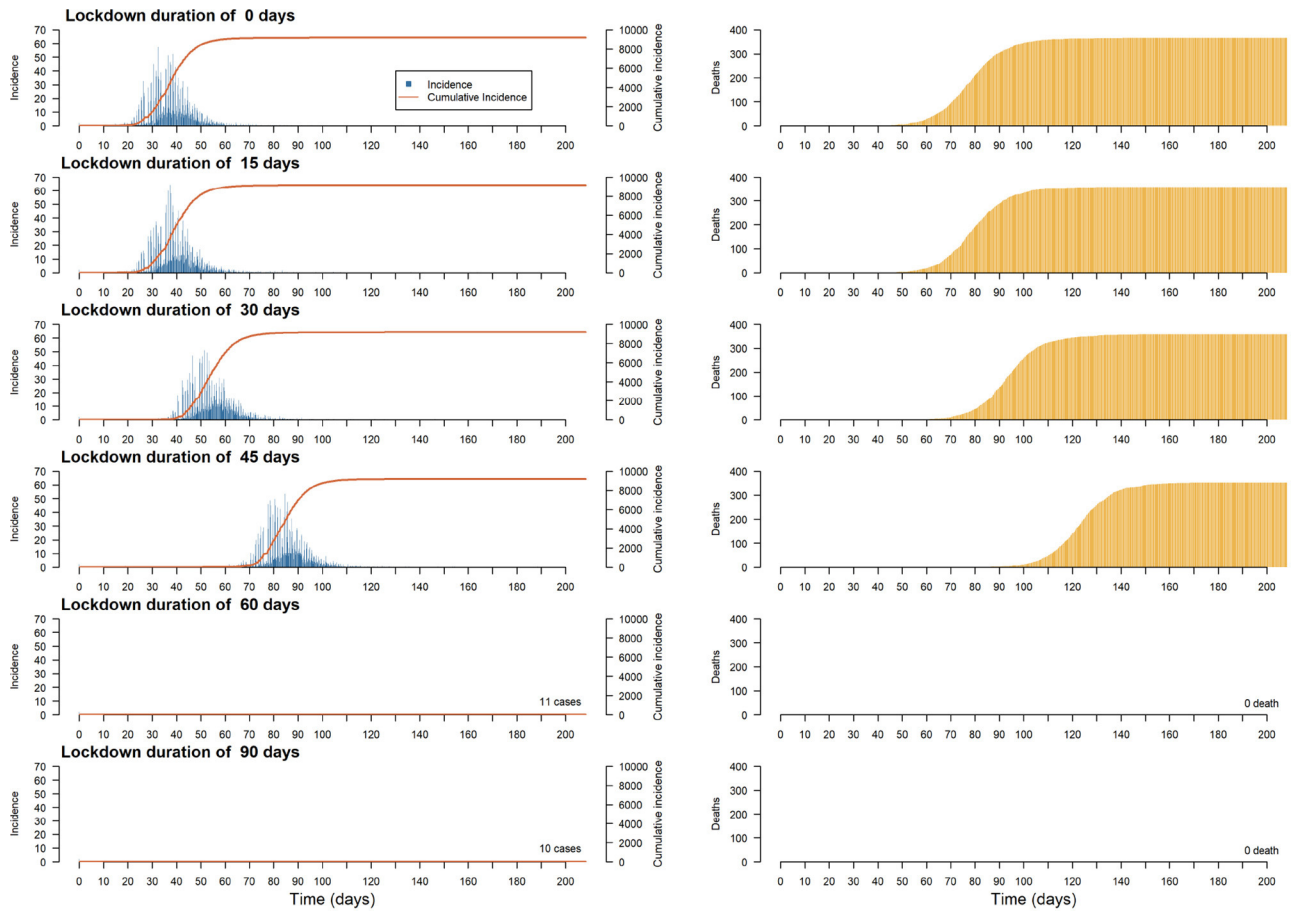


FIGURE 8 | Plots of the median of the incidence, cumulative incidence and deaths on the population per step for different lockdown durations.

of confirmed cases, significant mobility restrictions applied to 90% of the population (to take into account people who cannot work at home and who are essential for everyday activities). According to the French government, there have been ∼5,000 tests per day on average from the beginning of the epidemic, which is 4–5 times less than Korea or Germany. Regarding the lockdown, while it was one of the first countries to cancel major events, the closure of schools and non-essential economic activities occurred 14 days after the first deaths due to COVID-19, only preceding Great-Britain among the European countries (60).

- *A combination of policies similar to the one used in Malta*: no confinement for all the Individuals, but individuals belonging to risk groups (in the model: individuals over 50 years old) are required to stay at home.

In **Figure 9**, we depicted the policy consequences over the incidence (left column) and the number of casualties (right column). Only the policy involving a conjunction of mass testing and confirmed cases' household home confinement (similar to what South Korea implemented) have been able to contain the epidemic. However, the two other policies lead to specific mitigation outcomes: small sample testing in conjunction with heavy restriction on movement manages to delay and flatten the epidemic curve, while home containment directive toward at-risk people seems to lower the number of deaths.

All these experiences illustrate the characteristics and capabilities of COMOKIT. First of all, we have shown that, although the model is very stochastic, it is not very sensitive to this randomness, which allows us to launch explorations with a limited number (50) of replications. Second, we illustrated the ability of COMOKIT to compare different policies, for example by exploring the impact of the proportion of the population wearing a mask or the duration of a lock. Finally, we highlighted the expressive power of the model by implementing realistic policies close to those applied in three countries and by being able to compare their effectiveness at the scale and in the context of the Vietnamese commune used as a case study.

5. CONCLUSION AND PERSPECTIVES

In less than 3 months after its emergence in China, the COVID-19 pandemic spread to the entire world. In the absence of prior experience with this new disease, public health authorities were forced to experiment, in a short period of time and in a largely

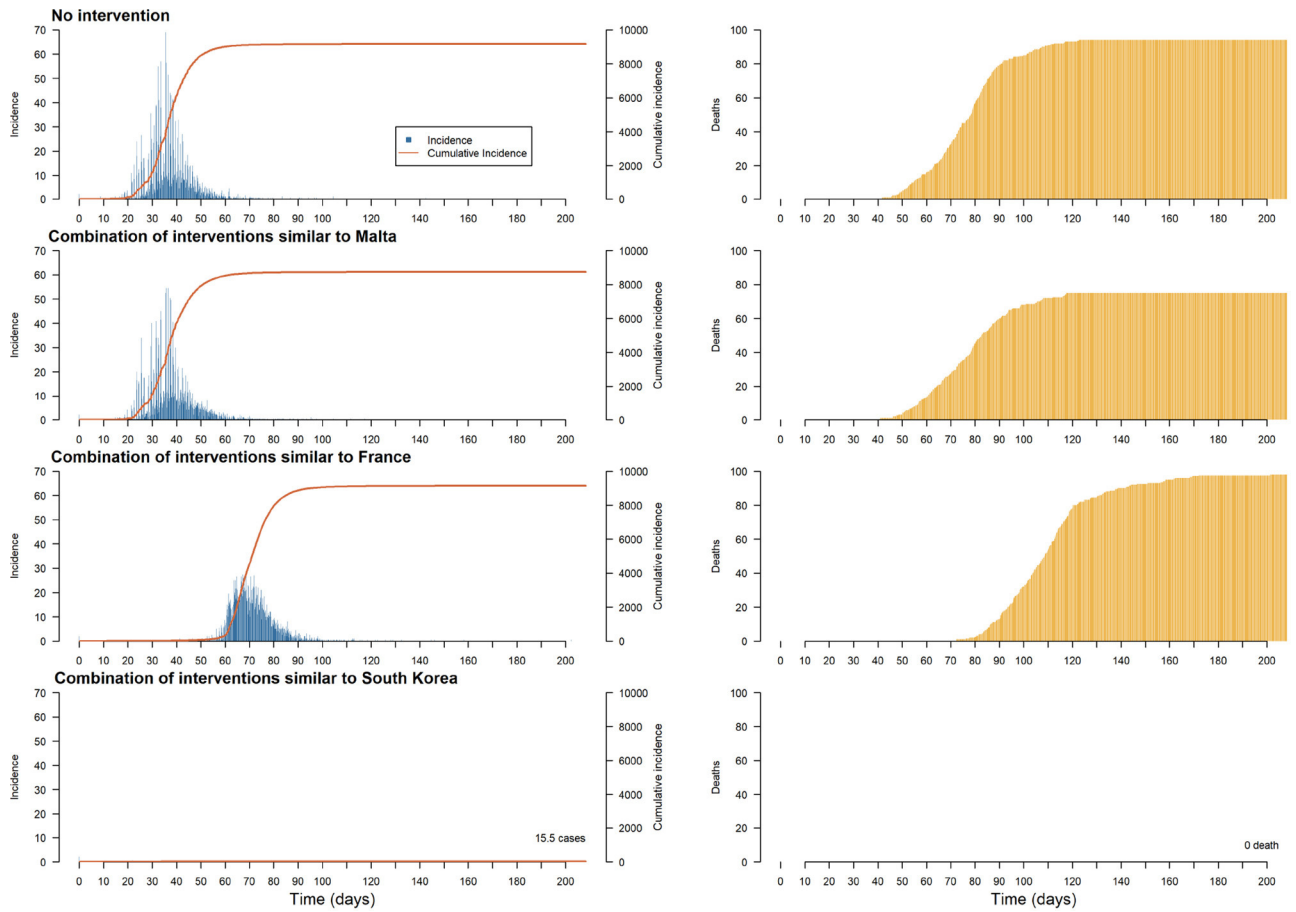


FIGURE 9 | Plots of the median of the incidence, cumulative incidence and deaths on the population per step for different realistic interventions (each with a duration of 60 days).

uninformed manner, with various combinations of interventions at different scales.

As the pandemic continues its progression, data are being collected from a variety of sources, allowing authorities to make adjustments to ongoing and planned interventions, but also revealing an urgent need for tools and methodologies to quickly analyse, understand, compare, and predict the effectiveness of responses to COVID-19 in different communities and contexts. In this perspective, computer modeling, and especially agent-based approaches, allows detailed *in silico* exploration of these responses prior to their potential implementation. In this paper, we presented an agent-based modeling software built on the GAMA platform called Covid-19 Modeling Kit (COMOKIT), designed to be generic, scalable, and thus portable in a variety of social and geographical contexts.

COMOKIT is an integrated model, presented in detail in section 3, which combines a direct person-to-person transmission sub-model, an environmental transmission sub-model across the built environment, a policy design sub-model, and a person mobility and activity model based on a 1-h time step agenda. As shown in section 4, COMOKIT offers many guarantees in terms of reproducibility of results and sensitivity to input parameters. In addition, as we have demonstrated by implementing and comparing different policies and policy combinations, COMOKIT is modular and flexible enough to allow modelers to represent different strategies and study their impacts under several social, epidemiological or economic scenarios. It should be noted that although it comes with a predefined set of policies and activities for individual agents (e.g., buying, studying, working, etc.), adapted to most contexts, it can easily be extended to new agents, policies or activities by editing the models written in GAML.

Thanks to this inner flexibility and genericity, and to the increasing availability of open data, new case studies can be processed in COMOKIT within a few hours, allowing it to be used in a variety of contexts and by a majority of decision-makers. In fact, as shown in section 3, the model can work with only a minimal (usually open) initial dataset: the built environment and administrative boundaries of the study area can be extracted from OpenStreetMap, while a statistically consistent synthetic population can be generated by the Genstar Toolkit from IPUMS

datasets. More accurate and sophisticated data can of course be mobilized to support the design of more complex models if required, and this can be done in a progressive and incremental fashion. This first version of COMOKIT (version v1.0, released in May 2020) has however some limitations that are already identified and that we think we can gradually remove with the help of other modelers:

- Scaling up: in computational terms, an agent-based approach will always be more expensive than an aggregate approach, not only in terms of execution time, but also in terms of the necessary replications (with respect to deterministic mathematical models). In its version 1.0, COMOKIT can reasonably (i.e., in less than 10 min on an average laptop with a graphical user interface enabled) simulate several months of pandemic fighting in cities with 10–20,000 inhabitants. Why take this standard? Quite simply because many users will test COMOKIT in this way and they should also be able to benefit from it. More serious experiments, varying more parameters and exploring different scenarios, will of course require scaling up. We are working on scaling up on two fronts: the first is to make it as easy as possible to use an HPC architecture from the simulator so that any user can access sufficient computing resources to run many replications or parallelize some of the operations of the simulations (66). This approach is the subject of a partnership with the EDF company, which has agreed to make its computing resources available (including the GAIA supercomputer); the second is to allow a more significant scaling-up of the model itself by implementing a hybrid approach (67–69) that is capable, dynamically, of aggregating individuals into groups of individuals according to different criteria (belonging to the same household, presence in the same space, sharing the same states, etc.) when this proves possible and relevant, in order to simulate much larger scales. As GAMA allows to couple computer models and mathematical models within the same simulation at different scales (70), this approach will not pose any technical problems, but it does raise quite interesting conceptual problems (71).
- The second limitation of the model is related to the assumptions made regarding the representation of group activities. So far, by design, no activities can be held outside a building and no group transportation is represented (for obvious reasons given the size of the initial case studies). This implies that agents cannot congregate outside buildings, nor can they congregate by chance; when they do congregate and have a chance to contaminate each other, it is because they are performing the same activity and/or are located in the same building. This strongly limits the representation of informal activities, such as markets or street restaurants, which are so common in Vietnam and other countries, outdoor public events (concerts, religious gatherings, etc.) or collective leisure activities (walks in pedestrian areas, parties, etc.), even though some of these activities (especially religious gatherings) are suspected to have contributed to the initial creation of clusters. Moving to larger scales will also, of course, require taking into account the transmission in public transport, from human to human during travel, but also through the environment, via the contamination of shared surfaces. These extensions are already planned for the next version of the model, but any new contribution is of course welcome!

The COVID-19 pandemic has resulted in countless casualties and contaminations, imposing massive public health campaigns, such as social isolation through widespread containment. The differences between countries and territories in terms of the occurrence of the virus and the number of victims are striking, as are the approaches of governments and their effectiveness in combating the pandemic. In such a context, it is important to recognize the increasing importance of data-based modeling approaches in the design of public health strategies. Platforms, such as COMOKIT can contribute to this effort, provided, as in this case, that they are open, transparent, easily explorable and testable, and above all built on sound theoretical and computational foundations.

DATA AVAILABILITY STATEMENT

The version of COMOKIT used in this paper is labeled V1.0. The source code of this version, together with the input dataset and all the experiments presented in this paper, are available in the repository of the project: https://github.com/COMOKIT/COMOKIT-Model. To be executed, COMOKIT requires GAMA, an open-source modeling and simulation platform, in its version 1.8.1. GAMA is available at: https://gama-platform.org. In order to make it as easy as possible for readers to run COMOKIT, an all-in-one release, including a Java virtual machine, the correct version of the GAMA platform and COMOKIT version 1.0 with all the datasets, is available here: https://github.com/COMOKIT/COMOKIT-Model/releases.

AUTHOR CONTRIBUTIONS

AD, BG, and PT were the principal designers of this document and were responsible for much of the writing. DP, NH, KC, and AB organized most of the experimental study to gather the study data and are responsible for the accuracy of data analysis and interpretation. PL performed the manuscript reviews. All authors have worked together, each in their own specialty, to design, and describe the research presented in the paper.

FUNDING

This work was supported by the ANRS (http://anrs.fr) through the funding of a COVID-19 SOUTH project (COV15) and EDF R&D (https://www.edf.fr/en/the-edf-group/who-we-are/activities/research-and-development) by providing access to their GAIA supercomputer to launch experiments.

ACKNOWLEDGMENTS

The authors would like to sincerely thank Dr. Marc Choisy of IRD and OUCRU for his help in the design of COMOKIT. They would also like to express their deep gratitude to Dr. François Sempé and the team at EDF R&D for making

COMOKIT run on their GAIA supercomputer. They finally wish to acknowledge the important contribution, in terms of data and directions, of Prof. Nguyen Ngoc Doanh (Thuyloi University) and Dr. Pham Quang Thai (NIHE), on behalf of the Rapid Response Team of the Vietnam Steering Committee against COVID-19.

REFERENCES

1. Zhu N, Zhang D, Wang W, Li X, Yang B, Song J, et al. A novel coronavirus from patients with pneumonia in China, 2019. *N Engl J Med.* (2020) 382:727–33. doi: 10.1056/NEJMoa2001017
2. Li Q, Guan X, Wu P, Wang X, Zhou L, Tong Y, et al. Early transmission dynamics in Wuhan, China, of novel coronavirus infected pneumonia. *N Engl J Med.* (2020) 382:1199–207. doi: 10.1056/NEJMoa2001316
3. For Knowledge Exchange Through SDG Partnerships USPC. *Korea's Rapid Innovations in the Time of COVID-19*. (2020). Available online at: http://www.undp.org/content/seoul_policy_center/en/home/presscenter/articles/2019/korea_s-rapid-innovations-in-the-time-of-covid-19.html
4. Flaxman S, Mishra S, Gandy A. *Report 13-Estimating the Number of Infections and the Impact of Non-pharmaceutical Interventions on COVID-19 in 11 European Countries*. London: Imperial College (2020).
5. Kamel Boulos MN, Geraghty EM. Geographical tracking and mapping of coronavirus disease COVID-19/severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) epidemic and associated events around the world: how 21st century GIS technologies are supporting the global fight against outbreaks and epidemics. *Int J Health Geograph.* (2020) 19:8. doi: 10.1186/s12942-020-00202-8
6. Ferguson N, Laydon D, Nedjati Gilani G, Imai N, Ainslie K, Baguelin M, et al. *Report 9: Impact of Non-pharmaceutical Interventions (NPIs) to Reduce COVID19 Mortality and Healthcare Demand*. London: Imperial College London (2020). Available online at: http://spiral.imperial.ac.uk/handle/10044/1/77482
7. Chow A, Hein AA, Kyaw WM. Unintended consequence: influenza plunges with public health response to COVID-19 in Singapore. *J Infect.* (2020) 81:e68–9. doi: 10.1016/j.jinf.2020.04.035
8. Nguyen T, Cuong Q, Kim A, Huong T, Nguyen H, Fox G, et al. Adapting a TB contact investigation strategy for COVID-19. *Int J Tuberc Lung Dis.* (2020) 24:169. doi: 10.5588/ijtld.20.0169
9. Thanh HN, Van TN, Thu HNT, Van BN, Thanh BD, Thu HPT, et al. Outbreak investigation for COVID-19 in northern Vietnam. *Lancet Infect Dis.* (2020) 20:535–6. doi: 10.1016/S1473-3099(20)30159-6
10. La VP, Pham TH, Ho MT, Nguyen MH, Nguyen KL, Vuong TT, et al. Policy response, social media and science journalism for the sustainability of the public health system amid the COVID-19 outbreak: the Vietnam lessons. *Sustainability.* (2020) 12:2931. doi: 10.3390/su12072931
11. Koo JR, Cook AR, Park M, Sun Y, Sun H, Lim JT, et al. Interventions to mitigate early spread of SARS-CoV-2 in Singapore: a modelling study. *Lancet Infect Dis.* (2020) 20:678–88. doi: 10.1016/S1473-3099(20)30162-6
12. Philippon D, Choisy M, Drogoul A, Gaudou B, Marilleau N, Taillandier P, et al. Exploring trade and health policies influence on dengue spread with an agent-based model. In: Nardin LG, Antunes L, editors. *Multi-Agent Based Simulation XVII. Lecture Notes in Computer Science*. Cham: Springer International Publishing (2017). p. 111–27. doi: 10.1007/978-3-319-67477-3_6
13. Walport M, Calder M, Craig C, Culley D, de Cani R, Donnelly C, et al. *Computational Modelling: Technological Futures*. London: Government Office for Science (2018).
14. Ferguson NM, Cummings DAT, Fraser C, Cajka JC, Cooley PC, Burke DS. Strategies for mitigating an influenza pandemic. *Nature.* (2006) 442:448–52. doi: 10.1038/nature04795
15. Keeling MJ, Rohani P. *Modeling Infectious Diseases in Humans and Animals*. Princeton, NJ: Princeton University Press (2007).
16. Di Domenico L, Pullano G, Sabbatini CE, Bolle PY, Colizza V. Expected impact of lockdown in le-de-France and possible exit strategies. *Infect Dis.* (2020). doi: 10.1101/2020.04.13.20063933
17. Hoertel N, Blachier M, Blanco C, Olfson M, Massetti M, Rico MS, et al. A stochastic agent-based model of the SARS-CoV-2 epidemic in France. *Nat Med.* (2020). doi: 10.1038/s41591-020-1001-6
18. Cliff OM, Harding N, Piraveenan M, Erten EY, Gambhir M, Prokopenko M. Investigating spatiotemporal dynamics and synchrony of influenza epidemics in Australia: an agent-based modelling approach. *Simul Modell Pract Theory.* (2018) 87:412–31. doi: 10.1016/j.simpat.2018.07.005
19. Chang SL, Harding N, Zachreson C, Cliff OM, Prokopenko M. Modelling transmission and control of the COVID-19 pandemic in Australia. *arXiv.* (2020) *arXiv [Preprint]* arXiv:2003.10218.
20. Bonabeau E. Agent-based modeling: Methods and techniques for simulating human systems. *Proc Natl Acad Sci USA.* (2002) 99:7280–7. doi: 10.1073/pnas.082080899
21. Mitchell M. *Complexity: A Guided Tour*. Oxford: Oxford University Press (2009).
22. Taillandier P, Gaudou B, Grignard A, Huynh QN, Marilleau N, Caillou P, et al. Building, composing and experimenting complex spatial models with the GAMA platform. *GeoInformatica.* (2019) 23:299–322. doi: 10.1007/s10707-018-00339-6
23. Drogoul A, Taillandier P, Gaudou B, Choisy M, Chapuis K, Huynh QN, et al. *Designing Social Simulation to (Seriously) Support Decision-Making: COMOKIT, An Agent-Based Modelling Toolkit to Analyse and Compare the Impacts of Public Health Interventions Against COVID-19*. Review of Artificial Societies and Social Simulation (2020). Available online at: https://rofasss.org/2020/04/27/comokit/
24. Wu JT, Leung K, Leung GM. Nowcasting and forecasting the potential domestic and international spread of the 2019-nCoV outbreak originating in Wuhan, China: a modelling study. *Lancet.* (2020) 395:689–97. doi: 10.1016/S0140-6736(20)30260-9
25. Gostic K, Gomez AC, Mummah RO, Kucharski AJ, Lloyd-Smith JO. Estimated effectiveness of symptom and risk screening to prevent the spread of COVID-19. *eLife.* (2020) 9:e55570. doi: 10.7554/eLife.55570.sa2
26. Ferretti L, Wymant C, Kendall M, Zhao L, Nurtay A, Abeler-Drner L, et al. Quantifying SARS-CoV-2 transmission suggests epidemic control with digital contact tracing. *Science.* (2020) 368:eabb6936. doi: 10.1101/2020.03.08.20032946
27. Prem K, Liu Y, Russell TW, Kucharski AJ, Eggo RM, Davies N, et al. The effect of control strategies to reduce social mixing on outcomes of the COVID-19 epidemic in Wuhan, China: a modelling study. *Lancet Public Health.* (2020) 5:E261–70. doi: 10.1101/2020.03.09.20033050
28. Chinazzi M, Davis JT, Ajelli M, Gioannini C, Litvinova M, Merler S, et al. The effect of travel restrictions on the spread of the 2019 novel coronavirus (COVID-19) outbreak. *Science.* (2020) 368:395–400. doi: 10.1126/science.aba9757
29. Moghadas SM, Shoukat A, Fitzpatrick MC, Wells CR, Sah P, Pandey A, et al. Projecting hospital utilization during the COVID-19 outbreaks in the United States. *Proc Natl Acad Sci USA.* (2020) 117:9122–6. doi: 10.1073/pnas.2004064117
30. Kissler SM, Tedijanto C, Goldstein E, Grad YH, Lipsitch M. Projecting the transmission dynamics of SARS-CoV-2 through the postpandemic period. *Science.* (2020) 368:860–8. doi: 10.1126/science.abb5793
31. Nickbakhsh S, Ho A, Marques DFP, McMenamin J, Gunson RN, Murcia PR. Epidemiology of seasonal coronaviruses: Establishing the context for COVID-19 emergence. *J Infect Dis.* (2020) 222:17–25. doi: 10.1101/2020.03.18.20037101
32. Dong Y, Mo X, Hu Y, Qi X, Jiang F, Jiang Z, et al. Epidemiology of COVID-19 among children in China. *Pediatrics.* (2020) 145:e20200702. doi: 10.1542/peds.2020-0702
33. Ludvigsson JF. Systematic review of COVID-19 in children shows milder cases and a better prognosis than adults. *Acta Paediatr.* (2020) 109:1088–95. doi: 10.1111/apa.15270
34. Ferguson N. Capturing human behaviour. *Nature.* (2007) 446:733. doi: 10.1038/446733a
35. Goldstein E, Lipsitch M. Temporal rise in the proportion of both younger adults and older adolescents among COVID-19 cases in Germany: evidence of lesser adherence to social distancing practices? *medRxiv.* (2020) 2020.04.08.20058719. doi: 10.1101/2020.04.08.20058719

36. Zhong BL, Luo W, Li HM, Zhang QQ, Liu XG, Li WT, et al. Knowledge, attitudes, and practices towards COVID-19 among Chinese residents during the rapid rise period of the COVID-19 outbreak: a quick online cross-sectional survey. *Int J Biol Sci.* (2020) 16:1745–52. doi: 10.7150/ijbs.45221
37. Al-Tawfiq JA, Rodriguez-Morales AJ. Super-spreading events and contribution to transmission of MERS, SARS, and COVID-19. *J Hosp Infect*. (2020) 105:111–112. doi: 10.1016/j.jhin.2020.04.002
38. Hellewell J, Abbott S, Gimma A, Bosse NI, Jarvis CI, Russell TW, et al. Feasibility of controlling COVID-19 outbreaks by isolation of cases and contacts. *Lancet Glob Health.* (2020) 8:e488–96. doi: 10.1016/S2214-109X(20)30074-7
39. Chin AWH, Chu JTS, Perera MRA, Hui KPY, Yen HL, Chan MCW, et al. Stability of SARS-CoV-2 in different environmental conditions. *Lancet Microbe.* (2020) 1:E10. doi: 10.1016/S2666-5247(20)30003-3
40. Jiang Y, Wang H, Chen Y, He J, Chen L, Liu Y, et al. Clinical data on hospital environmental hygiene monitoring and medical staff protection during the coronavirus disease 2019 outbreak. *medRxiv*. (2020) 2020.02.25.20028043. doi: 10.1101/2020.02.25.20028043
41. Ong SWX, Tan YK, Chia PY, Lee TH, Ng OT, Wong MSY, et al. Air, surface environmental, and personal protective equipment contamination by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) from a symptomatic patient. *JAMA*. (2020) 323:1610–2. doi: 10.1001/jama.2020.3227
42. Guo ZD, Wang ZY, Zhang SF, Li X, Li L, Li C, et al. Aerosol and surface distribution of severe acute respiratory syndrome coronavirus 2 in hospital wards, Wuhan, China, 2020. *Emerg Infect Dis.* (2020) 26:1583–91. doi: 10.3201/eid2607.200885
43. Bin SY, Heo JY, Song MS, Lee J, Kim EH, Park SJ, et al. Environmental contamination and viral shedding in MERS patients during MERS-CoV outbreak in South Korea. *Clin Infect Dis.* (2016) 62:755–60. doi: 10.1093/cid/civ1020
44. Dowell SF, Simmerman JM, Erdman DD, Wu JSJ, Chaovavanich A, Javadi M, et al. Severe acute respiratory syndrome coronavirus on hospital surfaces. *Clin Infect Dis.* (2004) 39:652–7. doi: 10.1086/422652
45. Bahl P, Doolan C, de Silva C, Chughtai AA, Bourouiba L, MacIntyre CR. Airborne or droplet precautions for health workers19 treating COVID-19? *J Infect Dis.* (2020). doi: 10.1093/infdis/jiaa189
46. van Doremalen N, Bushmaker T, Morris DH, Holbrook MG, Gamble A, Williamson BN, et al. Aerosol and surface stability of SARS-CoV-2 as compared with SARS-CoV-1. *N Engl J Med.* (2020) 382:1564–7. doi: 10.1056/NEJMc2004973
47. Correa-Martínez CL, Kampmeier S, Kümpers P, Schwierzeck V, Hennies M, Hafezi W, et al. A pandemic in times of global tourism: superspreading and exportation of COVID-19 cases from a ski area in Austria. *J Clin Microbiol.* (2020) 58:e00588-20. doi: 10.1128/JCM.00588-20
48. Wang Q, Shi N, Huang J, Cui T, Yang L, Ai J, et al. Effectiveness and cost-effectiveness of public health measures to control COVID-19: a modelling study. *medRxiv*. (2020) 2020.03.20.20039644. doi: 10.1101/2020.03.20.20039644
49. Grimm V, Berger U, DeAngelis DL, Polhill JG, Giske J, Railsback SF. The ODD protocol: a review and first update. *Ecol Modell.* (2010) 221:2760–8. doi: 10.1016/j.ecolmodel.2010.08.019
50. Chapuis K, Taillandier P, Renaud M, Drogoul A. Gen*: a generic toolkit to generate spatially explicit synthetic populations. *Int J Geograph Inform Sci.* (2018) 32:1194–210. doi: 10.1080/13658816.2018.1440563
51. Williamson P, Birkin M, Rees PH. The estimation of population microdata by using data from small area statistics and samples of anonymised records. *Environ Plann A.* (1998) 30:785–816. doi: 10.1068/a300785
52. Feng S, Shen C, Xia N, Song W, Fan M, Cowling BJ. Rational use of face masks in the COVID-19 pandemic. *Lancet Respir Med.* (2020) 8:434–6. doi: 10.1016/S2213-2600(20)30134-X
53. Leung NHL, Chu DKW, Shiu EYC, Chan KH, McDevitt JJ, Hau BJP, et al. Respiratory virus shedding in exhaled breath and efficacy of face masks. *Nat Med.* (2020) 26:676–80. doi: 10.1038/s41591-020-0843-2
54. He X, Lau EHY, Wu P, Deng X, Wang J, Hao X, et al. Temporal dynamics in viral shedding and transmissibility of COVID-19. *Nat Med.* (2020) 26:672–5. doi: 10.1038/s41591-020-1016-z
55. Mizumoto K, Kagaya K, Zarebski A, Chowell G. Estimating the asymptomatic proportion of coronavirus disease 2019 (COVID-19) cases on board the Diamond Princess cruise ship, Yokohama, Japan, 2020. *Eurosurveillance.* (2020) 25:2000180. doi: 10.2807/1560-7917.ES.2020.25.10.2000180
56. Hoehl S, Rabenau H, Berger A, Kortenbusch M, Cinatl J, Bojkova D, et al. Evidence of SARS-CoV-2 infection in returning travelers from Wuhan, China. *N Engl J Med.* (2020) 382:1278–80. doi: 10.1056/NEJMc2001899
57. Du Z, Xu X, Wu Y, Wang L, Cowling BJ, Meyers LA. The serial interval of COVID-19 from publicly reported confirmed cases. *medRxiv*. (2020) 2020.02.19.20025452. doi: 10.1101/2020.02.19.20025452
58. Li R, Pei S, Chen B, Song Y, Zhang T, Yang W, et al. Substantial undocumented infection facilitates the rapid dissemination of novel coronavirus (SARS-CoV2). *Science.* (2020) 368:489–93. doi: 10.1101/2020.02.14.20023127
59. Lewnard JA, Lo NC. Scientific and ethical basis for social-distancing interventions against COVID-19. *Lancet Infect Dis.* (2020) 20:631–3. doi: 10.1016/S1473-3099(20)30190-0
60. Roux J, Massonnaud C, Crépey P. COVID-19: one-month impact of the French lockdown on the epidemic burden. *Epidemiology.* (2020). doi: 10.1101/2020.04.22.20075705
61. Lau H, Khosrawipour V, Kocbach P, Mikolajczyk A, Schubert J, Bania J, et al. The positive impact of lockdown in Wuhan on containing the COVID-19 outbreak in China. *J Travel Med.* (2020) 27:taaa037. doi: 10.1093/jtm/taaa037
62. Guzzetta G, Riccardo F, Marziano V, Poletti P, Trentini F, Bella A, et al. The impact of a nation-wide lockdown on COVID-19 transmissibility in Italy. *arXiv.* (2020) *arXiv [Preprint].* arXiv:2004.12338.
63. Alvarez FE, Argente D, Lippi F. *A Simple Planning Problem for COVID-19 Lockdown*. National Bureau of Economic Research (2020). Available online at: http://www.nber.org/papers/w26981
64. Rossi R, Socci V, Talevi D, Mensi S, Niolu C, Pacitti F, et al. COVID-19 pandemic and lockdown measures impact on mental health among the general population in Italy. *Psychiatry Clin Psychol.* (2020) 11:790. doi: 10.1101/2020.04.09.20057802
65. Cadoni M, Gaeta G. How long does a lockdown need to be? *arXiv.* (2020) arXiv:2004.11633 [q-bio].
66. Scerri D, Hickmott S, Padgham L, Drogoul A. An architecture for modular distributed simulation with agent-based models. In: *9th International Conference on Autonomous Agents and Multiagent Systems (AAMAS)*. Toronto, ON: International Foundation for Autonomous Agents and Multiagent Systems (IFAAMAS) (2010). p. 541–8.
67. Drogoul A, Huynh NQ, Truong QC. Coupling environmental, social and economic models to understand land-use change dynamics in the Mekong Delta. *Front Environ Sci.* (2016) 4:19. doi: 10.3389/fenvs.2016.00019
68. Banos A, Corson N, Gaudou B, Laperrière V, Coyrehourcq SR. The importance of being hybrid for spatial epidemic models: a multi-scale approach. *Systems.* (2015) 3:309–29. doi: 10.3390/systems3040309
69. Nghi HQ, Nguyen-Huu T, Grignard A, Huynh HX, Drogoul A. Toward an agent-based and equation-based coupling framework. In: *International Conference on Nature of Computation and Communication*. Rach Gia: Springer (2016). p. 311–24. doi: 10.1007/978-3-319-46909-6_28
70. Vo DA, Drogoul A, Zucker JD. An operational meta-model for handling multiple scales in agent-based simulations. In: *2012 IEEE RIVF International Conference on Computing & Communication Technologies, Research, Innovation, and Vision for the Future*. Ho Chi Minh City: IEEE (2012). p. 1–6. doi: 10.1109/rivf.2012.6169849
71. Anh NTN, Daniel ZJ, Du NH, Drogoul A, An VD. A hybrid macro-micro pedestrians evacuation model to speed up simulation in road networks. In: *International Conference on Autonomous Agents and Multiagent Systems*. Taipei: Springer (2011). p. 371–83. doi: 10.1007/978-3-642-27216-5_28

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: frontiersin.org/about/contact

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership